Kongeriget Danmark

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Applicant:
(Name and address)

Genesto A/S
Damsholtevej 3
Gunderød
DK-2970 Hørsholm
Denmark

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Susanne Morsing

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Binding member towards Pneumococcus surface adhesin A protein (PsaA)

The present invention relates to a binding member comprising at least one binding domain capable of specifically binding Streptococcus pneumoniae surface adhesin A (PsaA) protein, in particular to a binding member having at least two binding domains, to the use of said binding members in diagnostic methods as well as for treatment.

Background

Streptococcus pneumoniae is one of the leading causes of life-threatening bacterial infection. In developing countries it has been estimated that several million children under 5 years of age will die of *S. pneumoniae* each year (3). In the industrialized world, the incidence of *S. pneumoniae* pneumonia is 5-10 per 100.000 persons and the case-fatality rate is 5-7%. *S. pneumoniae* meningitis occurs in 1-2 per 100.000 persons with a case-fatality of 30-40% (14). *S. pneumoniae* is one of the most frequent causes of bacteremia. *S. pneumoniae* is the most frequent organism isolated from children with otitis media. App. 75% of all children less than 6 years old will suffer from otitis media.

S. pneumoniae is a gram-positive bacteria that grows in pairs or short chains. The surface is composed of three layers: capsule, cell wall and plasma membrane. The capsule is the thickest layer and completely conceals the inner structures of growing *S. pneumoniae*. Polymers of repeating units of oligosaccharides (polysaccharides) dominant the capsule. Different serotypes contain ribitol, arabitinol or phosphorylcholine as part of their capsule, resulting in chemical structures that are serotype specific. The cell wall consists of peptidoglycan but also teichoic acid and lipoteichoic acid. The plasma membrane is a double phospholipid membrane that encompasses the cell and anchors various molecules to its surface (2).

At present 90 different types of *S. pneumoniae* are recognized based on the diversity of the *S. pneumoniae* capsule (26). The capsule is pivotal in the pathogenesis of *S. pneumoniae* infections. Antibodies raised against one capsular type offers protection from infection with this type but not against infection with other capsular

types. The current 23-valent polysaccharide vaccine offers protection from more than 60-85% of the most frequent serotypes.

The cell wall contains two polysaccarides, C-polysaccharide (C-PS) (teichoic acid and peptidoglycan) and F-antigen (lipoteichoic acid, Forssman antigen) (26). Other bacteria than *S. pneumoniae* contain C-PS, e.g. alpha-streptococci (12). F-antigen cross-reacts with streptococcal group C polysaccharide (29). Several antibodies to capsular polysaccharides cross-react with C-PS presumably because these are covalently linked. The protective role of anti-C-PS antibodies is controversial since some studies find them protective in mice (6) and others not (20;28). The lack of protection is believed to be caused by the capsular concealment of C-PS. Antibodies to C-PS may protect hosts infected with acapsular strains or bind to decaying *S. pneumoniae* that shed their capsule.
Immunization of mice with the F-antigen does not protect against *S. pneumoniae* infection (4).

Pneumococcal surface adhesin A (PsaA) is a 37-kDa surface protein. A monoclonal antibody towards PsaA reacted with 24 of 24 different encapsulated *S. pneumoniae* lysates and none of a number of other bacteria (23). RFLP analysis of the psaA gene from 80 strains representing 23 capsule serotypes showed they were highly conserved (24). Immunization of mice with PsaA provided protection against type 3 *S. pneumoniae* (32). Native PsaA is purified by standard methods (25;33). Recombinant PsaA can be expressed in a baculovirus vector system (10). Anti-rPsaA immune serum conferred protection in mice against *S. pneumoniae* serotype 6B compared to control mice (10). At least six different monoclonal antibodies have been reported (9;23), and suggested for diagnostic purposes, however treatment of Streptococcus pneumoniae associated diseases have not been suggested with these antibodies.

IgA to PsaA is detectable in saliva from children less than two years (193 of 261) and adults (17 of 17) (34). Anti-PsaA IgG was detectable by EIA in most children less than two years (872 of 1108) and most adults (262/325) (35). Seroconversion was correlated to carrier status, i.e. children who had had with *S. pneumoniae* cultured from nasopharyngeal or middle ear specimens were more likely to be anti-PsaA IgG positive.

Summary

The present invention relates to a binding member comprising at least one binding domain capable of specifically binding Streptococcus pneumoniae surface adhesin A (PsaA) protein, wherein the binding member is suitable for use in a pharmaceutical composition for preventing and treating diseases and disorders related to Streptococcus, in particular Streptococcus pneumoniae.

Accordingly, in one embodiment the invention relates to an isolated binding member comprising at least one binding domain capable of specifically binding Streptococcus pneumoniae surface adhesin A (PsaA) protein, said binding domain having a dissociation constant K_d for PsaA which is less than 1×10^{-6}. Preferably the binding member comprising the binding domain has the dissociation constant K_d defined above.

Due to the high binding strength the binding member is suitable for use in a pharmaceutical composition.

In another aspect the invention relates to an isolated binding member comprising at least a first binding domain and a second binding domain, said first binding domain being capable of specifically binding Streptococcus pneumoniae surface adhesin A (PsaA) protein.

The binding member according to the invention is preferably an antibody or a fragment of an antibody. The antibody may be produced by any suitable method known to the person skilled in the art, however it is preferred that at least a part of the binding member is produced through a recombinant method. Accordingly, the present invention relates in one aspect to an isolated nucleic acid molecule encoding at least a part of the binding member as defined above, as well as to a vector comprising the nucleic acid molecule defined above, and a host cell comprising the nucleic acid molecule defined above.

The invention further relates to a cell line engineered to express at least a part of the binding member as defined above, and more preferably engineered to express the whole binding member as defined above.

In a further aspect the invention relates to a method of detecting or diagnosing a disease or disorder associated with Pneumococcus in an individual comprising

- providing a biological sample from said individual,
- adding at least one binding member as defined above to said biological sample
- detecting binding members bound to said biological sample, thereby detecting or diagnosing the disease or disorder.

Also, in the method the invention further relates to a kit comprising at least one binding member as defined above, wherein said binding member is labelled, for use in a diagnostic method.

In yet another aspect the invention relates to a pharmaceutical composition comprising at least one binding member as defined above.

Furthermore, the invention relates to the use of a binding member as defined above for the production of a pharmaceutical composition for the treatment or prophylaxis of disorders or diseases associated with Streptococcus pneumoniae, such as pneumonia, meningitis and/or sepsis.

In yet a further aspect the invention relates to a method for treating or preventing an individual suffering from disorders or diseases associated with Streptococcus pneumoniae, such as pneumonia, meningitis and/or sepsis by administering an effective amount of a binding member as defined above.

Drawings

Figure 1. Schematic drawing of a Fab fragment.

Figure 2. Size exclusion HPLC profiles of the $F(ab')_2$ fragments of 88.53 (2a) and 5-9A7 (2b).

Figure 3. Size exclusion HPLC profile of the Fab' fragment of 88.53.

Figure 4. Size exclusion HPLC profile of the Fab' fragment of 5-9A7.

Figure 5. Size exclusion HPLC profile of 88.53 x 5-9A7 conjugation mixture.

Figure 6. Size exclusion HPLC profile of purified 88.53 x 5-9A7 bispecific antibody.

Figure 7. Size exclusion HPLC profiles of the $F(ab')_2$ fragments of 88.53 (7a) and 14A8 (7b).

Figure 8. Size exclusion HPLC profile of the Fab' fragment of 88.53.

Figure 9. Size exclusion HPLC profile of the Fab' fragment of 14A8.

Figure 10. Size exclusion HPLC profile of 88.53 x 14A8 conjugation mixture.

Figure 11. Size exclusion HPLC profile of purified 88.53 x 14A8 bispecific antibody.

Figure 12. Schematic picture of bispecific antibodies

Figure 13. Bispecific binding activity of the 88.53 x 5-9A7 bispecific antibody.

Figure 14. The 88.53 x 5-9A7 bispecific antibody binds to CD64 expressed by human CD64 transgenic mice.

Figure 15. PsaA amino acid sequence

Figure 16a Anti-PsaA 7-1G9 VK, wherein V-segment: L15 and J-segment: JK1

Figure 16b. Anti-PsaA 7-1G9 VH, wherein V-segment: 4-34, D-segment: unknown, and J-segment: JH4b

Figure 17a. Anti-PsaA 1-15E5 VK, V-segment: L6, and J-segment: JK4

Figure 17b. Anti-PsaA 1-15E5 VH, V-segment: 3-7, D-segment 3-10, J-segment: JH6b

Figure 18a. Anti-PsaA 9A7 VK, V-segment: L6, and J-segment: JK3

Figure 18b. Anti-PsaA 9A7 VH, V-segment: 3-7, D-segment: 3-10, and J-segment: JH6b

Figure 19. Four graphs showing the effect of anti-PsaA antibodies on Pneumococcus infection.

Figure 20. Table for results from in vitro tests.

Detailed description of the Invention

Definitions

Affinity: the strength of binding between receptors and their ligands, for example between an antibody and its antigen.

Avidity: The functional combining strength of an antibody with its antigen which is related to both the affinity of the reaction between the epitopes and paratopes, and the valencies of the antibody and antigen

Amino Acid Residue: An amino acid formed upon chemical digestion (hydrolysis) of a polypeptide at its peptide linkages. The amino acid residues described herein are preferably in the "L" isomeric form. However, residues in the "D" isomeric form can

be substituted for any L-amino acid residue, as long as the desired functional property is retained by the polypeptide. NH_2 refers to the free amino group present at the amino terminus of a polypeptide. COOH refers to the free carboxy group present at the carboxy terminus of a polypeptide. In keeping with standard polypeptide, abbreviations for amino acid residues are shown in the following Table of Correspondence:

TABLE OF CORRESPONDENCE

SYMBOL		
1-Letter	3-Letter	AMINO ACID
Y	Tyr	tyrosine
G	Gly	glycine
F	Phe	phenylalanine
M	Met	methionine
A	Ala	alanine
S	Ser	serine
I	Ile	isoleucine
L	Leu	leucine
T	Thr	threonine
V	Val	valine
P	Pro	proline
K	Lys	lysine
H	His	histidine
Q	Gln	glutamine
E	Glu	glutamic acid
Z	Glx	Glu and/or Gln
W	Trp	tryptophan
R	Arg	arginine
D	Asp	aspartic acid
N	Asn	asparagine
B	Asx	Asn and/or Asp
C	Cys	cysteine
X	Xaa	Unknown or other

It should be noted that all amino acid residue sequences represented herein by for-

mulae have a left-to-right orientation in the conventional direction of amino terminus to carboxy terminus. In addition, the phrase "amino acid residue" is broadly defined to include the amino acids listed in the Table of Correspondence and modified and unusual amino acids. Furthermore, it should be noted that a dash at the beginning or end of an amino acid residue sequence indicates a peptide bond to a further sequence of one or more amino acid residues or a covalent bond to an amino-terminal group such as NH_2 or acetyl or to a carboxy-terminal group such as COOH.

Antibody: The term antibody in its various grammatical forms is used herein to refer to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules of the compositions of this invention, i.e., molecules that contain an antibody combining site or paratope. Exemplary antibody molecules are intact immunoglobulin molecules, substantially intact immunoglobulin molecules and portions of an immunoglobulin molecule, including those portions known in the art as Fab, Fab', $F(ab')_2$ and Fv. A schematic drawing of Fab is shown in Figure 1. The term "antibody" as used herein is also intended to include human, single chain and humanized antibodies, as well as binding fragments of such antibodies or modified versions of such antibodies, such as multispecific, bispecific and chimeric molecules having at least one antigen binding determinant derived from an antibody molecule.

Antibody Combining Site: An antibody combining site is that structural portion of an antibody molecule comprised of a heavy and light chain variable and hypervariable regions that specifically binds (immunoreacts with) an antigen. The term immunoreact in its various forms means specific binding between an antigenic determinant-containing molecule and a molecule containing an antibody combining site such as a whole antibody molecule or a portion thereof. Alternatively, an antibody combining site is known as an antigen binding site.

Base Pair (bp): A partnership of adenine (A) with thymine (T), or of cytosine (C) with guanine (G) in a double stranded DNA molecule. In RNA, uracil (U) is substituted for thymine.

Binding member: a polypeptide that can bind to an epitope on a Streptococcus pneumoniae protein, in particular capable of binding specifically to PsaA.

Binding domain: An antigen binding site which specifically binds an antigen. A binding member may be multispecific and contain two or more binding domains which specifically bind two immunologically distinct antigens.

Chimeric antibody: An antibody in which the variable regions are from one species of animal and the constant regions are from another species of animal. For example, a chimeric antibody can be an antibody having variable regions which derive from a mouse monoclonal antibody and constant regions which are human.

Complementary Bases: Nucleotides that normally pair up when DNA or RNA adopts a double stranded configuration.

Complementarity determining region or CDR: Regions in the V-domains of an antibody that together form the antibody recognizing and binding domain.

Complementary Nucleotide Sequence: A sequence of nucleotides in a single-stranded molecule of DNA or RNA that is sufficiently complementary to that on another single strand to specifically hybridize to it with consequent hydrogen bonding.

Conserved: A nucleotide sequence is conserved with respect to a preselected (reference) sequence if it non-randomly hybridizes to an exact complement of the preselected sequence.

Conservative Substitution: The term conservative substitution as used herein denotes the replacement of an amino acid residue by another, biologically similar residue. Examples of conservative substitutions include the substitution of one hydrophobic residue such as isoleucine, valine, leucine or methionine for another, or the substitution of one polar residue for another, such as the substitution of arginine for lysine, glutamic for aspartic acids, or glutamine for asparagine, and the like. The term conservative substitution also includes the use of a substituted amino acid in place of an unsubstituted parent amino acid provided that molecules having the substituted polypeptide also have the same function.

Constant Region or constant domain or C-domain: Constant regions are those structural portions of an antibody molecule comprising amino acid residue se-

quences within a given isotype which may contain conservative substitutions therein. Exemplary heavy chain immunoglobulin constant regions are those portions of an immunoglobulin molecule known in the art as CH1, CH2, CH3, CH4 and CH5. An exemplary light chain immunoglobulin constant region is that portion of an immunoglobulin molecule known in the art as C_L.

Dissociation constant, Kd: a measure to describe the strength of binding (or affinity or avidity) between receptors and their ligands, for example an antibody and its antigen.The smaller Kd the stronger binding.

Downstream: Further along a DNA sequence in the direction of sequence transcription or read out, that is traveling in a 3'- to 5'-direction along the non-coding strand of the DNA or 5'- to 3'-direction along the RNA transcript.

Duplex DNA: A double-stranded nucleic acid molecule comprising two strands of substantially complementary polynucleotides held together by one or more hydrogen bonds between each of the complementary bases present in a base pair of the duplex. Because the nucleotides that form a base pair can be either a ribonucleotide base or a deoxyribonucleotide base, the phrase "duplex DNA" refers to either a DNA-DNA duplex comprising two DNA strands (ds DNA), or an RNA-DNA duplex comprising one DNA and one RNA strand.

Fusion Polypeptide: A polypeptide comprised of at least two polypeptides and a linking sequence to operatively link the two polypeptides into one continuous polypeptide. The two polypeptides linked in a fusion polypeptide are typically derived from two independent sources, and therefore a fusion polypeptide comprises two linked polypeptides not normally found linked in nature.

Fv: dual chain antibody fragment containing both a V_H and a V_L.

Gene: A nucleic acid whose nucleotide sequence codes for an RNA or polypeptide. A gene can be either RNA or DNA.

Human antibody framework: A molecule having an antigen binding site and essentially all remaining immunoglobulin-derived parts of the molecule derived from a human immunoglobulin.

Humanised antibody framework: A molecule having an antigen binding site derived from an immunoglobulin from a non-human species, whereas some or all of the remaining immunoglobulin-derived parts of the molecule is derived from a human immunoglobulin. The antigen binding site may comprise: either a complete variable domain from the non-human immunoglobulin fused onto one or more human constant domains; or one or more of the complementarity determining regions (CDRs) grafted onto appropriate human framework regions in the variable domain. In a humanized antibody, the CDRs can be from a mouse monoclonal antibody and the other regions of the antibody are human.

Hybridization: The pairing of substantially complementary nucleotide sequences (strands of nucleic acid) to form a duplex or heteroduplex by the establishment of hydrogen bonds between complementary base pairs. It is a specific, i.e. non-random, interaction between two complementary polynucleotides that can be competitively inhibited.

Immunoglobulin: The serum antibodies, including IgG, IgM, IgA, IgE and IgD.

Immunoglobulin isotypes: The names given to the Ig which have different H chains, the names are IgG ($IgG_{1,2,3,4}$), IgM, IgA ($IgA_{1,2}$), sIgA, IgE, IgD.

Immunologically distinct: The phrase immunologically distinct refers to the ability to distinguish between two polypeptides on the ability of an antibody to specifically bind one of the polypeptides and not specifically bind the other polypeptide.

Individual: A living animal or human in need of susceptible to a condition, in particular an infectious disease" as defined below. The subject is an organism possessing leukocytes capable of responding to antigenic stimulation and growth factor stimulation. In preferred embodiments, the subject is a mammal, including humans and non-human mammals such as dogs, cats, pigs, cows, sheep, goats, horses, rats, and mice. In the most preferred embodiment, the subject is a human.

Infectious disease: a disorder caused by one or more species of Streptococcus, in particular Streptococcus pneumoniae.

Isolated: is used to describe the various binding members, polypeptides and nucleotides disclosed herein, that has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials that would typically interfere with diagnostic or therapeutic uses for the polypeptide, and may include enzymes, hormones, and other proteinaceous or non-proteinaceous solutes. In preferred embodiments, the polypeptide will be purified.

Label and indicating means: refer in their various grammatical forms to single atoms and molecules that are either directly or indirectly involved in the production of a detectable signal to indicate the presence of a complex

Monoclonal Antibody: The phrase monoclonal antibody in its various grammatical forms refers to a population of antibody molecules that contains only one species of antibody combining site capable of immunoreacting with a particular antigen. A monoclonal antibody thus typically displays a single binding affinity for any antigen with which it immunoreacts. A monoclonal antibody may contain an antibody molecule having a plurality of antibody combining sites, each immunospecific for a different antigen, e.g., a bispecific monoclonal antibody.

Multimeric: A polypeptide molecule comprising more than one polypeptide. A multimer may be dimeric and contain two polypeptides and a multimer may be trimeric and contain three polypeptides. Multimers may be homomeric and contain two or more identical polypeptides or a multimer may be heteromeric and contain two or more nonidentical polypeptides.

Nucleic Acid: A polymer of nucleotides, either single or double stranded.

Nucleotide: A monomeric unit of DNA or RNA consisting of a sugar moiety (pentose), a phosphate, and a nitrogenous heterocyclic base. The base is linked to the sugar moiety via the glycosidic carbon (1' carbon of the pentose) and that combina-

tion of base and sugar is a nucleoside. When the nucleoside contains a phosphate group bonded to the 3' or 5' position of the pentose it is referred to as a nucleotide. A sequence of operatively linked nucleotides is typically referred to herein as a "base sequence" or "nucleotide sequence", and their grammatical equivalents, and is represented herein by a formula whose left to right orientation is in the conventional direction of 5'-terminus to 3'-terminus.

Nucleotide Analog: A purine or pyrimidine nucleotide that differs structurally from A, T, G, C, or U, but is sufficiently similar to substitute for the normal nucleotide in a nucleic acid molecule.

Pneumococcus: is used synonymously with Streptococcus pneumoniae.

Polyclonal antibody: Polyclonal antibodies is a mixture of antibody molecules recognising a specific given antigen, hence polyclonal antibodies may recognise different epitopes within said antigen.

Polynucleotide: A polymer of single or double stranded nucleotides. As used herein "polynucleotide" and its grammatical equivalents will include the full range of nucleic acids. A polynucleotide will typically refer to a nucleic acid molecule comprised of a linear strand of two or more deoxyribonucleotides and/or ribonucleotides. The exact size will depend on many factors, which in turn depends on the ultimate conditions of use, as is well known in the art. The polynucleotides of the present invention,include primers, probes, RNA/DNA segments, oligonucleotides or "oligos" (relatively short polynucleotides), genes, vectors, plasmids, and the like.

Polypeptide: The phrase polypeptide refers to a molecule comprising amino acid residues which do not contain linkages other than amide linkages between adjacent amino acid residues.

Receptor: A receptor is a molecule, such as a protein, glycoprotein and the like, that can specifically (non-randomly) bind to another molecule.

Recombinant DNA (rDNA) molecule: A DNA molecule produced by operatively linking two DNA segments. Thus, a recombinant DNA molecule is a hybrid DNA mole-

cule comprising at least two nucleotide sequences not normally found together in nature. rDNA's not having a common biological origin, i.e., evolutionarily different, are said to be "heterologous".

Specificity: The term specificity refers to the number of potential antigen binding sites which immunoreact with (specifically bind to) a given antigen in a polypeptide. The polypeptide may be a single polypeptide or may be two or more polypeptides joined by disulfide bonding. A polypeptide may be monospecific and contain one or more antigen binding sites which specifically bind an antigen or a polypeptide may be bispecific and contain two or more antigen binding sites which specifically bind two immunologically distinct antigens. Thus, a polypeptide may contain a plurality of antigen binding sites which specifically bind the same or different antigens.

Serotype: Identification of bacteria within species of Streptococcus, that consist of many strains differing from one another in a variety of characteristics. Commonly used characteristics defining serotypes are particular antigenic molecules.

Single Chain Antibody or scFv: The phrase single chain antibody refers to a single polypeptide comprising one or more antigen binding sites. Furthermore, although the H and L chains of an Fv fragment are encoded by separate genes, they may be linked either directly or via a peptide, for example a synthetic linker can be made that enables them to be made as a single protein chain (known as single chain antibody, sAb; Bird et al. 1988 Science 242:423-426; and Huston et al. 1988 PNAS 85:5879-5883) by recombinant methods. Such single chain antibodies are also encompassed within the term "antibody", and may be utilized as binding determinants in the design and engineering of a multispecific binding molecule.

Upstream: In the direction opposite to the direction of DNA transcription, and therefore going from 5' to 3' on the non-coding strand, or 3' to 5' on the mRNA.

Valency: The term valency refers to the number of potential antigen binding sites, i.e. binding domains, in a polypeptide. A polypeptide may be monovalent and contain one antigen binding site or a polypeptide may be bivalent and contain two antigen binding sites. Additionally, a polypeptide may be tetravalent and contain four antigen binding sites. Each antigen binding site specifically binds one antigen. When

a polypeptide comprises more than one antigen binding site, each antigen binding site may specifically bind the same or different antigens. Thus, a polypeptide may contain a plurality of antigen binding sites and therefore be multivalent and a polypeptide may specifically bind the same or different antigens.

V-domain: Variable domain are those structural portions of an antibody molecule comprising amino acid residue sequences forming the antigen binding sites. An exemplary light chain immunoglobulin variable region is that portion of an immunoglobulin molecule known in the art as V_L.

V_L: Variable domain of the light chain

V_H: Variable domain of the heavy chain

Vector: A rDNA molecule capable of autonomous replication in a cell and to which a DNA segment, e.g., gene or polynucleotide, can be operatively linked so as to bring about replication of the attached segment. Vectors capable of directing the expression of genes encoding for one or more polypeptides are referred to herein as "expression vectors". Particularly important vectors allow cloning of cDNA (complementary DNA) from mRNAs produced using reverse transcriptase.

Description

As described above, the present invention relates to binding members, in particular antibodies or fragments thereof capable of specifically recognising and binding to a Streptococcus pneumoniae protein, more specifically to Pneumococcus surface adhesin A protein, PsaA. The binding members according to the invention are particularly useful in the treatment of diseases caused by Streptococcus pneumoniae, as well as for being employed in diagnostic methods and kits for detecting the bacteria. The Penumococcus surface adhesin A protein is preferably a polypeptide having the amino acid sequence shown Figure 15.

Thus, the binding member according to the invention should preferably be immunologically active, for example as an antibody, such as being capable of binding to

an antigen and presenting the antigen to immunoactive cells, thereby facilitating phagocytosis of said antigen.

In particular the binding member is an antibody, such as any suitable antibody known in the art, in partucular antibodies as defined herein, such as antibodies or immunologically active fragments of antibodies, or single chain antibodies. Antibody molecules are typically Y-shaped molecules whose basic unit consist of four polypeptides, two identical heavy chains and two identical light chains, which are covalently linked together by disulfide bonds. Each of these chains is folded in discrete domains. The C-terminal regions of both heavy and light chains are conserved in sequence and are called the constant regions, also known as C-domains. The N-terminal regions, also known as V-domains, are variable in sequence and are responsible for the antibody specificity. The antibody specifically recognizes and binds to an antigen mainly through six short complementarity-determining regions located in their V-domains (see Fig. 1).

The antibodies according to the invention are especially useful, since they have a strong affinity towards the PsaA.

Accordingly, the binding members according to the invention have a binding domain having a dissociation constant K_d for PsaA which is less than 1×10^{-6} M. More preferably the dissociation constant K_d for PsaA which is less than 1×10^{-7}M, more preferably less than 1×10^{-8}M, more preferably less than 5×10^{-8}M, more preferably less than 1×10^{-9}M, more preferably less than 5×10^{-9}M more preferably less than 1×10^{-10}M.

The affinity of the binding member towards the PsaA is preferably measured as described in Example 2.

The binding member is preferably an isolated binding member as defined above, and more preferably an isolated, pure binding member.

Complementarity-determining regions

Without being bound by theory it is believed that the high binding strength is caused by incorporating into the binding domain an amino acid sequence having one or more of the following motifs of the sequences shown below.

More specifically the binding domain preferably comprises a CDR1 region comprising a sequence selected from

SEQ ID NO 1: CDR1 OF AMINO ACID SEQUENCE IN FIG. 16A
SEQ ID NO 5: CDR1 OF AMINO ACID SEQUENCE IN FIG. 16A
SEQ ID NO 9: CDR1 OF AMINO ACID SEQUENCE IN FIG. 16A
SEQ ID NO 13: CDR1 OF AMINO ACID SEQUENCE IN FIG. 16A
SEQ ID NO 17 CDR1 OF AMINO ACID SEQUENCE IN FIG. 16A
SEQ ID NO 21: CDR1 OF AMINO ACID SEQUENCE IN FIG. 16A

And/or the binding domain preferably comprises a CDR2 region comprising a sequence selected from

SEQ ID NO 2: CDR2 OF AMINO ACID SEQUENCE IN FIG. 16A
SEQ ID NO 6: CDR2 OF AMINO ACID SEQUENCE IN FIG. 16B
SEQ ID NO 10: CDR2 OF AMINO ACID SEQUENCE IN FIG. 17A
SEQ ID NO 14: CDR2 OF AMINO ACID SEQUENCE IN FIG. 17B
SEQ ID NO 18: CDR2 OF AMINO ACID SEQUENCE IN FIG. 18A
SEQ ID NO 22: CDR2 OF AMINO ACID SEQUENCE IN FIG. 18B

And/or the binding domain preferably comprises a CDR3 region comprising a sequence selected from

SEQ ID NO 3: CDR3 OF AMINO ACID SEQUENCE IN FIG. 16A
SEQ ID NO 7: CDR3 OF AMINO ACID SEQUENCE IN FIG. 16B
SEQ ID NO 11: CDR3 OF AMINO ACID SEQUENCE IN FIG. 17A
SEQ ID NO 15: CDR3 OF AMINO ACID SEQUENCE IN FIG. 17B
SEQ ID NO 19: CDR3 OF AMINO ACID SEQUENCE IN FIG. 18A
SEQ ID NO 23: CDR3 OF AMINO ACID SEQUENCE IN FIG. 18B

More preferably the variable part of the binding domain comprises a sequence selected from

SEQ ID NO 4: AMINO ACID SEQUENCE IN FIG. 16A
SEQ ID NO 8: AMINO ACID SEQUENCE IN FIG. 16B
SEQ ID NO 12: AMINO ACID SEQUENCE IN FIG. 17A
SEQ ID NO 16: AMINO ACID SEQUENCE IN FIG. 17B
SEQ ID NO 20: AMINO ACID SEQUENCE IN FIG. 18A
SEQ ID NO 24: AMINO ACID SEQUENCE IN FIG. 18B

or a homologue thereof, wherein a homologue is as defined elsewhere herein.

More preferably the binding domain comprises at least one of the amino acid sequence sets selected from the group of

the amino acid sequence sets SEQ ID NO 1 or a homologue thereof, SEQ ID NO 2 or a homologue thereof, and SEQ ID NO 3 or a homologue thereof, or
the amino acid sequence sets SEQ ID NO 5 or a homologue thereof, SEQ ID NO 6 or a homologue thereof, and SEQ ID NO 7 or a homologue thereof, or
the amino acid sequence sets SEQ ID NO 9 or a homologue thereof, SEQ ID NO 10 or a homologue thereof, and SEQ ID NO 11 or a homologue thereof, or
the amino acid sequence sets SEQ ID NO 13 or a homologue thereof, SEQ ID NO 14 or a homologue thereof, and SEQ ID NO 15 or a homologue thereof, or
the amino acid sequence sets SEQ ID NO 17 or a homologue thereof, SEQ ID NO 18 or a homologue thereof, and SEQ ID NO 19 or a homologue thereof, or
the amino acid sequence sets SEQ ID NO 21 or a homologue thereof, SEQ ID NO 22 or a homologue thereof, and SEQ ID NO 23 or a homologue thereof.

In the amino acid sequence sets above, the amino acid sequences are preferably arranged in the binding domain as CDR1, CDR2 and CDR3, i.e. spaced apart by other amino acid sequences.

The homology of any one of the homologues described above preferably confers the binding domain comprising one or more homologues with a dissociation constant K_d for PsaA as defined above.

Identity and homology

The term "identity" or "homology" shall be construed to mean the percentage of amino acid residues in the candidate sequence that are identical with the residue of a corresponding sequence to which it is compared, after aligning the sequences and introducing gaps, if necessary to achieve the maximum percent identity for the entire sequence, and not considering any conservative substitutions as part of the sequence identity. Neither N- or C-terminal extensions nor insertions shall be construed as reducing identity or homology. Methods and computer programs for the alignment are well known in the art. Sequence identity may be measured using sequence analysis software (e.g., Sequence Analysis Software Package, Genetics

Computer Group, University of Wisconsin Biotechnology Center, 1710 University Ave., Madison, Wis. 53705). This software matches similar sequences by assigning degrees of homology to various substitutions, deletions, and other modifications.

A homologue of one or more of the sequences specified herein may vary in one or more amino acids as compared to the sequences defined, but is capable of performing the same function, i.e. a homologue may be envisaged as a functional equivalent of a predetermined sequence.

As described above a homologue of any of the predetermined sequences herein may be defined as:

i) homologues comprising an amino acid sequence capable of recognising an antigen also being recognised by the predetermined amino acid sequence, and/or

ii) homologues comprising an amino acid sequence capable of binding selectively to an antigen, wherein said antigen is also bound selectively by a predetermined sequence, and/or

iii) homolouges having a substantially similar or higher binding affinity to PsaA as a binding domain comprising a predetermined sequence, such as SEQ ID NO: 4.

Examples of homologues comprises one or more conservative amino acid substitutions including one or more conservative amino acid substitutions within the same group of predetermined amino acids, or a plurality of conservative amino acid substitutions, wherein each conservative substitution is generated by substitution within a different group of predetermined amino acids.

Homologues may thus comprise conservative substitutions independently of one another, wherein at least one glycine (Gly) of said homologue is substituted with an amino acid selected from the group of amino acids consisting of Ala, Val, Leu, and Ile, and independently thereof, homologues, wherein at least one of said alanines (Ala) of said homologue thereof is substituted with an amino acid selected from the group of amino acids consisting of Gly, Val, Leu, and Ile, and independently thereof,

homologues, wherein at least one valine (Val) of said homologue thereof is substituted with an amino acid selected from the group of amino acids consisting of Gly, Ala, Leu, and Ile, and independently thereof, homologues thereof, wherein at least one of said leucines (Leu) of said homologue thereof is substituted with an amino acid selected from the group of amino acids consisting of Gly, Ala, Val, and Ile, and independently thereof, homologues thereof, wherein at least one isoleucine (Ile) of said homologues thereof is substituted with an amino acid selected from the group of amino acids consisting of Gly, Ala, Val and Leu, and independently thereof, homologues thereof wherein at least one of said aspartic acids (Asp) of said homologue thereof is substituted with an amino acid selected from the group of amino acids consisting of Glu, Asn, and Gln, and independently thereof, homologues thereof, wherein at least one of said phenylalanines (Phe) of said homologues thereof is substituted with an amino acid selected from the group of amino acids consisting of Tyr, Trp, His, Pro, and preferably selected from the group of amino acids consisting of Tyr and Trp, and independently thereof, homologues thereof, wherein at least one of said tyrosines (Tyr) of said homologues thereof is substituted with an amino acid selected from the group of amino acids consisting of Phe, Trp, His, Pro, preferably an amino acid selected from the group of amino acids consisting of Phe and Trp, and independently thereof, homologues thereof, wherein at least one of said arginines (Arg) of said fragment is substituted with an amino acid selected from the group of amino acids consisting of Lys and His, and independently thereof, homologues thereof, wherein at least one lysine (Lys) of said homologues thereof is substituted with an amino acid selected from the group of amino acids consisting of Arg and His, and independently thereof, homologues thereof, wherein at least one of said aspargines (Asn) of said homologues thereof is substituted with an amino acid selected from the group of amino acids consisting of Asp, Glu, and Gln, and independently thereof, homologues thereof, wherein at least one glutamine (Gln) of said homologues thereof is substituted with an amino acid selected from the group of amino acids consisting of Asp, Glu, and Asn, and independently thereof, homologues thereof, wherein at least one proline (Pro) of said homologues thereof is substituted with an amino acid selected from the group of amino acids consisting of Phe, Tyr, Trp, and His, and independently thereof, homologues thereof, wherein at least one of said cysteines (Cys) of said homologues thereof is substituted with an amino acid selected from the group of amino acids consisting of Asp, Glu, Lys, Arg, His, Asn, Gln, Ser, Thr, and Tyr.

Conservative substitutions may be introduced in any position of a preferred predetermined sequence. It may however also be desirable to introduce non-conservative substitutions, particularly, but not limited to, a non-conservative substitution in any one or more positions.

A non-conservative substitution leading to the formation of a functionally equivalent homologueof the sequences herein would for example i) differ substantially in polarity, for example a residue with a non-polar side chain (Ala, Leu, Pro, Trp, Val, Ile, Leu, Phe or Met) substituted for a residue with a polar side chain such as Gly, Ser, Thr, Cys, Tyr, Asn, or Gln or a charged amino acid such as Asp, Glu, Arg, or Lys, or substituting a charged or a polar residue for a non-polar one; and/or ii) differ substantially in its effect on polypeptide backbone orientation such as substitution of or for Pro or Gly by another residue; and/or iii) differ substantially in electric charge, for example substitution of a negatively charged residue such as Glu or Asp for a positively charged residue such as Lys, His or Arg (and vice versa); and/or iv) differ substantially in steric bulk, for example substitution of a bulky residue such as His, Trp, Phe or Tyr for one having a minor side chain, e.g. Ala, Gly or Ser (and vice versa).

Substitution of amino acids may in one embodiment be made based upon their hydrophobicity and hydrophilicity values and the relative similarity of the amino acid side-chain substituents, including charge, size, and the like. Exemplary amino acid substitutions which take various of the foregoing characteristics into consideration are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine.

In a preferred embodiment the binding domain comprises a homologue having an amino acid sequence at least 60 % homologous to a sequence selected from SEQ ID NO 1, SEQ ID NO 2, SEQ ID NO 3 and SEQ ID NO 4.

More preferably the homology is at least 65 %, such as at least 70 % homologous, such as at least 75 % homologous, such as at least 80 % homologous, such as at least 85 % homologous, such as at least 90 % homologous, such as at least 95 % homologous, such as at least 98 % homologous to a sequence selected from SEQ ID NO 1, SEQ ID NO 2, SEQ ID NO 3 and SEQ ID NO 4.

In a more preferred embodiment the percentages mentioned above relates to the identity of the sequence of a homologue as compared to a sequence selected from SEQ ID NO 1, SEQ ID NO 2, SEQ ID NO 3 and SEQ ID NO 4.

Serotypes

As described above, 90 different serotypes of Streptococcus pneumoniae have been identified. It is preferred that the binding member according to this invention is capable of binding PsaA from two or more different Pneumococcus serotypes, such as from three or more different Pneumococcus serotypes, such as from four or more different Pneumococcus serotypes, such as from five or more different Pneumococcus serotypes. Most preferably the binding member according to the invention is capable of recognising and binding Pneumococcus from essentially all serotypes.

Monoclonal/polyclonal antibodies

In one embodiment of the invention the binding member is an antibody, wherein the antibody may be a polyclonal or a monoclonal antibody derived from a mammal or mixtures of monoclonal antibodies. In a preferred embodiment the binding member is a monoclonal antibody or a fragment thereof. The antibody may be any kind of antibody, however it is preferably a IgG antibody. More preferably the antibody is a IgG1 antibody or a fragment thereof.

Monoclonal antibodies (Mab's) are antibodies, wherein every antibody molecule are similar and thus recognises the same epitope. Monoclonal antibodies are in general produced by a hybridoma cell line. Methods of making monoclonal antibodies and antibody-synthesizing hybridoma cells are well known to those skilled in the art. Antibody producing hybridomas may for example be prepared by fusion of an antibody producing B lymphocyte with an immortalized cell line.

A monoclonal antibody can be produced by the following steps. In all procedures, an animal is immunized with an antigen such as a protein (or peptide thereof) as described above for preparation of a polyclonal antibody. The immunization is typically accomplished by administering the immunogen to an immunologically competent

mammal in an immunologically effective amount, i.e., an amount sufficient to produce an immune response. Preferably, the mammal is a rodent such as a rabbit, rat or mouse. The mammal is then maintained on a booster schedule for a time period sufficient for the mammal to generate high affinity antibody molecules as described. A suspension of antibody-producing cells is removed from each immunized mammal secreting the desired antibody. After a sufficient time to generate high affinity antibodies, the animal (e.g., mouse) is sacrificed and antibody-producing lymphocytes are obtained from one or more of the lymph nodes, spleens and peripheral blood. Spleen cells are preferred, and can be mechanically separated into individual cells in a physiological medium using methods well known to one of skill in the art. The antibody-producing cells are immortalized by fusion to cells of a mouse myeloma line. Mouse lymphocytes give a high percentage of stable fusions with mouse homologous myelomas, however rat, rabbit and frog somatic cells can also be used. Spleen cells of the desired antibody-producing animals are immortalized by fusing with myeloma cells, generally in the presence of a fusing agent such as polyethylene glycol. Any of a number of myeloma cell lines suitable as a fusion partner are used with to standard techniques, for example, the P3-NS1/1-Ag4-1, P3-x63-Ag8.653 or Sp2/O-Ag14 myeloma lines, available from the American Type Culture Collection (ATCC), Rockville, Md.

Monoclonal antibodies can also be generated by other methods well known to those skilled in the art of recombinant DNA technology. An alternative method, referred to as the "combinatorial antibody display" method, has been developed to identify and isolate antibody fragments having a particular antigen specificity, and can be utilized to produce monoclonal antibodies.

Polyclonal antibodies is a mixture of antibody molecules recognising a specific given antigen, hence polyclonal antibodies may recognise different epitopes within said antigen. In general polyclonal antibodies are purified from serum of a mammal, which previously has been immunized with the antigen. Polyclonal antibodies may for example be prepared by any of the methods described in Antibodies: A Laboratory Manual, By Ed Harlow and David Lane, *Cold Spring Harbor Laboratory Press, 1988.* Polyclonal antibodies may be derived from any suitable mammalian species, for example from mice, rats, rabbits, donkeys, goats, and sheep.

Specificity

The binding member may be monospecific towards the PsaA protein, wherein specificity towards the PsaA protein means that the binding member immunoreacts with PsaA protein. In another embodiment the binding member is bispecific or multispecific having at least one portion being specific towards the PsaA protein.

Monovalent antibodies

The monospecific binding member may be monovalent, i.e. having only one binding domain.

For a monovalent antibody, the immunoglobulin constant domain amino acid residue sequences comprise the structural portions of an antibody molecule known in the art as CH1, CH2, CH3 and CH4. Preferred are those binding members which are known in the art as C_L. Preferrred C_L polypeptides are selected from the group consisting of C_{kappa}. and C_{lambda}.

Furthermore, insofar as the constant domain can be either a heavy or light chain constant domain (C_H or C_L, respectively), a variety of monovalent binding member compositions are contemplated by the present invention. For example, light chain constant domains are capable of disulfide bridging to either another light chain constant domain, or to a heavy chain constant domain. In contrast, a heavy chain constant domain can form two independent disulfide bridges, allowing for the possibility of bridging to both another heavy chain and to a light chain, or to form polymers of heavy chains.

Thus, in another embodiment, the invention contemplates a composition comprising a monovalent polypeptide wherein the constant chain domain C has a cysteine residue capable of forming at least one disulfide bridge, and where the composition comprises at least two monovalent polypeptides covalently linked by said disulfide bridge.

In preferred embodiments, the constant chain domain C can be either C_L or C_H.

Where C is C_L, the C_L polypeptide is preferably selected from the group consisting of C_{kappa} and C_{lambda}.

In another embodiment, the invention contemplates a binding member composition comprising a monovalent polypeptide as above except where C is C_L having a cysteine residue capable of forming a disulfide bridge, such that the composition contains two monovalent polypeptides covalently linked by said disulfide bridge.

Multivalent

In another embodiment of the invention the binding member is a multivalent binding member having at least two binding domains. The binding domains may have specificity for the same ligand or for different ligands.

Multispecificity, including bispecificity

In a preferred embodiment the instant invention relates to multispecific binding members, which have affinity for and are capable of binding at least two different entities. Multispecific binding members can include bispecific binding members.

In one embodiment the multispecific molecule is a bispecific antibody (BsAb), which carries at least two different binding domains, at least one of which is of antibody origin.

A bispecific molecule of the invention can also be a single chain bispecific molecule, such as a single chain bispecific antibody, a single chain bispecific molecule comprising one single chain antibody and a binding domain, or a single chain bispecific molecule comprising two binding domains. Multispecific molecules can also be single chain molecules or may comprise at least two single chain molecules.

The multispecific, including bispecific antibodies may be produced by any suitable manner known to the person skilled in the art.

The traditional approach to generate bispecific whole antibodies was to fuse two hybridoma cell lines each producing an antibody having the desired specificity. Be-

cause of the random association of immunoglobulin heavy and light chains, these hybrid hybridomas produce a mixture of up to 10 different heavy and light chain combinations, only one of which is the bispecific antibody. Therefore, these bispecific antibodies have to be purified with cumbersome procedures, which considerably decrease the yield of the desired product.

Alternative approaches include in-vitro linking of two antigen specificities by chemical cross-linking of cysteine residues either in the hinge or via a genetically introduced C-terminal Cys as described above. An improvement of such in vitro assembly was achieved by using recombinant fusions of Fab's with peptides that promote formation of heterodimers. However, the yield of bispecific product in these methods is far less than 100%.

A more efficient approach to produce bivalent or bispecific antibody fragments, not involving in vitro chemical assembly steps, was described by Holliger et al. (1993). This approach takes advantage of the observation that scFv's secreted from bacteria are often present as both monomers and dimers. This observation suggested that the V_H and VL of different chains can pair, thus forming dimers and larger complexes. The dimeric antibody fragments, also named "diabodies" by Hollinger et al., in fact are small bivalent antibody fragments that assembled in vivo. By linking the V_H and VL of two different antibodies 1 and 2, to form "cross-over" chains V_H 1VL 2 and V_H 2-VL 1, the dimerisation process was shown to reassemble both antigen-binding sites. The affinity of the two binding sites was shown to be equal to the starting scFv's, or even to be 10-fold increased when the polypeptide linker covalently linking V_H and VL was removed, thus generating two proteins each consisting of a V_H directly and covalently linked to a VL not pairing with the V_H. This strategy of producing bispecific antibody fragments was also described in several patent applications. Patent application WO 94/09131 (SCOTGEN LTD; priority date Oct. 15, 1992) relates to a bispecific binding protein in which the binding domains are derived from both a V_H and a VL region either present at two chains or linked in an scFv, whereas other fused antibody domains, e.g. C-terminal constant domains, are used to stabilise the dimeric constructs. Patent application WO 94/13804 (CAMBRIDGE ANTIBODY TECHNOLOGY/MEDICAL RESEARCH COUNCIL; first priority date Dec. 4, 1992) relates to a polypeptide containing a V_H and a VL which are in-

capable of associating with each other, whereby the V-domains can be connected with or without a linker.

Mallender and Voss, 1994 (also described in patent application WO 94/13806; DOW CHEMICAL CO; priority date Dec. 11, 1992) reported the in vivo production of a single-chain bispecific antibody fragment in E. coli. The bispecificity of the bivalent protein was based on two previously produced monovalent scFv molecules possessing distinct specificities, being linked together at the genetic level by a flexible polypeptide linker. Traditionally, whenever single-chain antibody fragments are referred to, a single molecule consisting of one heavy chain linked to one (corresponding) light chain in the presence or absence of a polypeptide linker is implicated. When making bivalent or bispecific antibody fragments through the 'diabody' approach (Holliger et al., (1993) and patent application WO 94/09131) or by the 'double scFv' approach (Mallender and Voss, 1994 and patent application WO 94/13806), again the V_H is linked to a (the corresponding) VL .

The multispecific molecules described above can be made by a number of methods. For example, all specificities can be encoded in the same vector and expressed and assembled in the same host cell. This method is particularly useful where the multispecific molecule is a mAb X mAb, mAb X Fab, Fab X $F(ab')_2$ or ligand X Fab fusion protein. Various other methods for preparing bi- or multivalent antibodies are described for example described in U.S. Pat. Nos. 5,260,203; 5,455,030; 4,881,175; 5,132,405; 5,091,513; 5,476,786; 5,013,653; 5,258,498; and 5,482,858.

By using a bispecific or multispecific binding member according to the invention the invention offers several advantages as compared to monospecific/monovalent binding members.

A bispecific/multispecific binding member has a first binding domain capable of specifically recognising and binding a Streptococcus protein, in particular PsaA, whereas the other binding domain(s) may be used for other purposes:

In one embodiment at least one other binding domain is used for binding to a Streptococcus protein, such as binding to another epitope on the same Streptococcus protein as compared to the first binding domain. Thereby specificity for the

Streptococcus species may be increased as well as increase of avidity of the binding member.

In another embodiment the at least one other binding domain may be used for specifically binding a mammalian cell, such as a human cell. It is preferred that the at least other binding domain is capable of binding an immunoactive cell, such as a leucocyte, a macrophage, a lymphocyte, a basophilic cell, and/or an eosinophilic cell, in order to increase the effect of the binding member in a therapeutic method. This may be accomplished by establishing that the at least one other binding domain is capable of specifically binding a mammalian protein, such as a human protein, such as a protein selected from any of the cluster differentiation proteins (CD), in particular CD64 and/or CD89. A method for producing bispecific antibodies having CD64 specificity is described in US 6,0713517 to Medarex, Inc.

An "effector cell" as used herein refers to an immune cell which is a leukocyte or a lymphocyte. Specific effector cells express specific Fc receptors and carry out specific immune functions. For example, monocytes, macrophages, neutrophils, eosinophils, and lymphocytes which express CD89 receptor are involved in specific killing of target cells and presenting antigens to other components of the immune system, or binding to cells that present antigens.

Humanised antibody framework

It is not always desirable to use non-human antibodies for human therapy, since the non-human "foreign" epitopes may elicit immune response in the individual to be treated. To eliminate or minimize the problems associated with non-human antibodies, it is desirable to engineer chimeric antibody derivatives, i.e., "humanized" antibody molecules that combine the non-human Fab variable region binding determinants with a human constant region (Fc). Such antibodies are characterized by equivalent antigen specificity and affinity of the monoclonal and polyclonal antibodies described above, and are less immunogenic when administered to humans, and therefore more likely to be tolerated by the individual to be treated.

Accordingly, in one embodiment the binding member has a binding domain carried on a humanised antibody framework, also called a humanised antibody.

Humanised antibodies are in general chimeric antibodies comprising regions derived from a human antibody and regions derived from a non-human antibody, such as a rodent antibody. Humanisation (also called Reshaping or CDR-grafting) is a well-established technique for reducing the immunogenicity of monoclonal antibodies (mAbs) from xenogeneic sources (commonly rodent), increasing the homology to a human immunoglobulin, and for improving their activation of the human immune system. Thus, humanized antibodies are typically human antibodies in which some CDR residues and possibly some framework residues are substituted by residues from analogous sites in rodent antibodies.

It is further important that humanized antibodies retain high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of certain residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is maximized, although it is the the CDR residues that directly and most substantially influence antigen binding.

One method for humanising MAbs related to production of chimeric antibodies in which an antigen binding site comprising the complete variable domains of one antibody are fused to constant domains derived from a second antibody, preferably a human antibody. Methods for carrying out such chimerisation procedures are for example described in EP-A-0 120 694 (Celltech Limited), EP-A-0 125 023 (Genentech Inc.), EP-A-0 171 496 (Res. Dev. Corp. Japan), EP-A-0173494 (Stanford University) and EP-A-0 194 276 (Celltech Limited). A more complex form of humanisation of an antibody involves the re-design of the variable region domain so that the

amino acids constituting the non-human antibody binding site are integrated into the framework of a human antibody variable region (Jones et al., 1986).

The humanized antibody of the present invention may be made by any method capable of replacing at least a portion of a CDR of a human antibody with a CDR derived from a non-human antibody. Winter describes a method which may be used to prepare the humanized antibodies of the present invention (UK Patent Application GB 2188638A, filed on Mar. 26, 1987), the contents of which is expressly incorporated by reference. The human CDRs may be replaced with non-human CDRs using oligonucleotide site-directed mutagenesis as described in the examples below.

As an example the humanized antibody of the present invention may be made as described in the brief explanation below. The humanized antibodies of the present invention may be produced by the following process:

(a) constructing, by conventional techniques, an expression vector containing an operon with a DNA sequence encoding an antibody heavy chain in which the CDRs and such minimal portions of the variable domain framework region that are required to retain antibody binding specificity are derived from a non-human immunoglobulin, and the remaining parts of the antibody chain are derived from a human immunoglobulin, thereby producing the vector of the invention;

(b) constructing, by conventional techniques, an expression vector containing an operon with a DNA sequence encoding a complementary antibody light chain in which the CDRs and such minimal portions of the variable domain framework region that are required to retain donor antibody binding specificity are derived from a non-human immunoglobulin, and the remaining parts of the antibody chain are derived from a human immunoglobulin, thereby producing the vector of the invention;

(c) transfecting the expression vectors into a host cell by conventional techniques to produce the transfected host cell of the invention; and

(d) culturing the transfected cell by conventional techniques to produce the humanised antibody of the invention.

The host cell may be cotransfected with the two vectors of the invention, the first vector containing an operon encoding a light chain derived polypeptide and the second vector containing an operon encoding a heavy chain derived polypeptide. The two vectors contain different selectable markers, but otherwise, apart from the antibody heavy and light chain coding sequences, are preferably identical, to ensure, as far as possible, equal expression of the heavy and light chain polypeptides. Alternatively, a single vector may be used, the vector including the sequences encoding both the light and the heavy chain polypeptides. The coding sequences for the light and heavy chains may comprise cDNA or genomic DNA or both.

The host cell used to express the altered antibody of the invention may be either a bacterial cell such as Escherichia coli, or a eukaryotic cell. In particular a mammalian cell of a well defined type for this purpose, such as a myeloma cell or a Chinese hamster ovary cell may be used.

The general methods by which the vectors of the invention may be constructed, transfection methods required to produce the host cell of the invention and culture methods required to produce the antibody of the invention from such host cells are all conventional techniques. Likewise, once produced, the humanized antibodies of the invention may be purified according to standard procedures as described below.

Human antibody framework

In a more preferred embodiment the invention relates to a binding member, wherein the binding domain is carried by a human antibody framework, i.e. wherein the antibodies have a greater degree of human peptide sequences than do humanised antibodies.

Human mAb antibodies directed against human proteins can be generated using transgenic mice carrying the complete human immune system rather than the mouse system. Splenocytes from these transgenic mice immunized with the antigen of interest are used to produce hybridomas that secrete human mAbs with specific affinities for epitopes from a human protein (see, e.g., Wood et al. International Application WO 91/00906, Kucherlapati et al. PCT publication WO 91/10741; Lonberg

et al. International Application WO 92/03918; Kay et al. International Application 92/03917; Lonberg, N. et al. 1994 Nature 368:856-859; Green, L. L. et al. 1994 Nature Genet. 7:13-21; Morrison, S. L. et al. 1994 Proc. Natl. Acad. Sci. USA 81:6851-6855; Bruggeman et al. 1993 Year Immunol 7:33-40; Tuaillon et al. 1993 PNAS 90:3720-3724; Bruggeman et al. 1991 Eur J Immunol 21:1323-1326).

Such transgenic mice are available from Abgenix, Inc., Fremont, Calif., and Medarex, Inc., Annandale, N.J. It has been described that the homozygous deletion of the antibody heavy-chain joining region (IH) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits et al., Proc. Natl. Acad. Sci. USA 90:2551 (1993); Jakobovits et al., Nature 362:255-258 (1993); Bruggermann et al., Year in Immunol. 7:33 (1993); and Duchosal et al. Nature 355:258 (1992). Human antibodies can also be derived from phage-display libraries (Hoogenboom et al ., J. Mol. Biol. 227: 381 (1991); Marks et al., J. Mol. Biol. 222:581-597 (1991); Vaughan, et al., Nature Biotech 14:309 (1996)).

In a preferred embodiment the antibodies are produced by the method described in Example 1.

Fragments

In one embodiment of the invention the binding member is a fragment of an antibody, preferably an antigen binding fragment or a variable region. Examples of antibody fragments useful with the present invention include Fab, Fab', $F(ab')_2$ and Fv fragments. Papain digestion of antibodies produces two identical antigen binding fragments, called the Fab fragment, each with a single antigen binding site, and a residual "Fc" fragment, so-called for its ability to crystallize readily. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen binding fragments which are capable of cross-linking antigen, and a residual other fragment (which is termed pFc'). Additional fragments can include diabodies, linear antibodies, single-chain antibody molecules, and multispecific antibodies formed from antibody fragments.

The antibody fragments Fab, Fv and scFv differ from whole antibodies in that the antibody fragments carry only a single antigen-binding site. Recombinant fragments with two binding sites have been made in several ways, for example, by chemical cross-linking of cysteine residues introduced at the C-terminus of the VH of an Fv (Cumber et al., 1992), or at the C-terminus of the VL of an scFv (Pack and Pluckthun, 1992), or through the hinge cysteine residues of Fab's (Carter et al., 1992).

Preferred antibody fragments retain some or essential all the ability of an antibody to selectively binding with its antigen or receptor. Some preferred fragments are defined as follows:

(1) Fab is the fragment that contains a monovalent antigen-binding fragment of an antibody molecule. A Fab fragment can be produced by digestion of whole antibody with the enzyme papain to yield an intact light chain and a portion of one heavy chain.

(2) Fab' is the fragment of an antibody molecule and can be obtained by treating whole antibody with pepsin, followed by reduction, to yield an intact light chain and a portion of the heavy chain. Two Fab' fragments are obtained per antibody molecule. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxyl terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region.

(3) $(Fab')_2$ is the fragment of an antibody that can be obtained by treating whole antibody with the enzyme pepsin without subsequent reduction. $F(ab')_2$ is a dimer of two Fab' fragments held together by two disulfide bonds.

(4) Fv is the minimum antibody fragment that contains a complete antigen recognition and binding site. This region consists of a dimer of one heavy and one light chain variable domain in a tight, non-covalent association (V_H -V_L dimer). It is in this configuration that the three CDRs of each variable domain interact to define an antigen binding site on the surface of the V_H -V_L dimer. Collectively, the six CDRs confer antigen binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an an-

tigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

In one embodiment of the present invention the antibody is a single chain antibody ("SCA"), defined as a genetically engineered molecule containing the variable region of the light chain, the variable region of the heavy chain, linked by a suitable polypeptide linker as a genetically fused single chain molecule. Such single chain antibodies are also refered to as "single-chain Fv" or "sFv" antibody fragments. Generally, the Fv polypeptide further comprises a polypeptide linker between the V_H and VL domains that enables the sFv to form the desired structure for antigen binding.

The antibody fragments according to the invention may be produced in any suitable manner known to the person skilled in the art. Several microbial expression systems have already been developed for producing active antibody fragments, e.g. the production of Fab in various hosts, such as E. coli (Better et al., 1988, Skerra and Pluckthun, 1988, Carter et al., 1992), yeast (Horwitz et al., 1988), and the filamentous fungus Trichoderma reesei (Nyyssonen et al., 1993) has been described. The recombinant protein yields in these alternative systems can be relatively high (1-2 g/l for Fab secreted to the periplasmic space of E. coli in high cell density fermentation, see Carter et al., 1992), or at a lower level, e.g. about 0.1 mg/l for Fab in yeast in fermenters (Horwitz et al., 1988), and 150 mg/l for a fusion protein CBHI-Fab and 1 mg/l for Fab in Trichoderma in fermenters (Nyyssonen et al., 1993) and such production is very cheap compared to whole antibody production in mammalian cells (hybridoma, myeloma, CHO).

The fragments can be produced as Fab's or as Fv's, but additionally it has been shown that a VH and a VL can be genetically linked in either order by a flexible polypeptide linker, which combination is known as an scFv.

Isolated nucleic acid molecule/vector/host cell

In one aspect the invention relates to an isolated nucleic acid molecule encoding at least a part of the binding member as defined above. In one embodiment the nucleic acid molecule encodes a light chain and another nucleic acid encodes a heavy chain. The two nucleic acid molecule may be separate or they may be fused into

one nucleic acid molecule, optionally spaced apart by a linker sequence. In particular in relation to antibody fragments the nucleic acid molecule may encode the whole binding member, however dependant on the design of the binding member this may also be relevant for some larger binding members. The nucleic acid molecule preferably is a DNA sequence, more preferably a DNA sequence comprising in its upstream end regulatory elements promoting the expression of the binding member once the nucleic acid molecule is arranged in a host cell.

Accordingly, in one embodiment the invention relates to a polynucleotide selected from the group consisting of

i) a polynucleotide comprising a sequence selected from the nucleotide sequence of Figure 16a, Figure 16b, Figure 17a, Figure 17b, Figure 18a, and Figure 18b,

ii) a polynucleotide encoding a binding member comprising one or more of the amino acid sequence selected from the group of Figure 16a, Figure 16b, Figure 17a, Figure 17b, Figure 18a, and Figure 18b,

iii) a polynucleotide encoding a fragment of a polypeptide encoded by polynucleotides i), wherein said fragment

a) is capable of recognising an antigen also being recognised by the binding member of ii), and/or

b) is capable of binding selectively to an antigen, wherein said antigen is also bound selectively by by the binding member of ii),, and/or

c) has a substantially similar or higher binding affinity to PsaA as a binding domain comprising a predetermined sequence, such as SEQ ID NO: 4, SEQ ID NO: 8, SEQ ID NO: 12, SEQ ID NO: 16, SEQ ID NO: 20, SEQ ID NO: 24,

iv) a polynucleotide, the complementary strand of which hybridizesm under stringent conditions, with a polynucleotide as defined in any of i), ii), iii), and encodes a polypeptide as defined in iii),

v) a polynucleotide comprising a nucleotide sequence which is degenerate to the nucleotide sequence of a polynucleotide as defined in any of i) – iv),

and the complementary strand of such a polynucleotide.

The invention further relates to a vector comprising the nucleic acid molecule as defined above, either one vector per nucleic acid, or two or more nucleic acids in the same vector. The vector preferably comprises a nucleotide sequence which regulates the expression of the antibody encoded by the nucleic acid molecule.

In yet another aspect the invention relates to a host cell comprising the nucleic acid molecule as defined above.

Also, the invention relates to a cell line engineered to express the binding member as defined above, this cell line for example being a hybridoma of a murine lymphocyte and an immortalised cell line. The cell line may be any suitable cell line, however the cell line P3 is preferred. In another embodiment a CHO cell line is preferred.

Purification of binding members

After production the binding members according to the invention are preferably purified. The method of purification used is dependent upon several factors including the purity required, the source of the antibody, the intended use for the antibody, the species in which the antibody was produced, the class of the antibody and, when the antibody is a monoclonal antibody, the subclass of the antibody.

Any suitable conventional methods of purifying polypeptides comprising antibodies include precipitation and column chromatography and are well known to one of skill

in the purification arts, including cross-flow filtration, ammonium sulphate precipitation, affinity column chromatography, gel electrophoresis and the like may be used.

The method of purifying an antibody with an anti-immunoglobulin antibody can be either a single purification procedure or a sequential purification procedure. Methods of single and sequential purification are well known to those in the purification arts. In a single-step purification procedure, the antibody is specifically bound by a single anti-immunoglobulin antibody. Non-specifically bound molecules are removed in a wash step and the specifically bound molecules are specifically eluted. In a sequential purification procedure, the antibody is specifically bound to a first anti-immunoglobulin antibody, non-specifically bound molecules are removed in a wash step, and the specifically bound molecules are specifically eluted. The eluant from the first anti-immunoglobulin antibody is then specifically bound to a second anti-immunoglobulin antibody. The non-specifically bound molecules are removed in a wash step, and the specifically bound molecules are specifically eluted. In a preferred embodiment, the antibody is sequentially purified by a first and second anti-immunoglobulin antibody selected from the group consisting of antibodies which specifically bind heavy and light chain constant regions. In a more preferred em

A commonly used method of purification is affinity chromatography in which the antibody to be purified is bound by protein A, protein G or by an anti-immunoglobulin antibody. Another method of affinity chromatography, which is well known to those of skill in the art, is the specific binding of the antibody to its respective antigen.

In particular for purifying a multispecific, including a bispecific antibody, a sequential purification procedure may be used, wherein the bispecific antibody comprising two or more variable domains is specifically bound to a first antigen and then to a second antigen.

In an alternative embodiment, a bispecific antibody comprising two or more variable regions is purified by sequential purification by specifically binding the antibody to a first antigen in a first purification step and to a second antigen in a second purification step.

The method of purifying an antibody with an anti-immunoglobulin antibody can be

either a single purification procedure or a sequential purification procedure. Methods of single and sequential purification are well known to those in the purification arts. In a single-step purification procedure, the antibody is specifically bound by a single anti-immunoglobulin antibody. Non-specifically bound molecules are removed in a wash step and the specifically bound molecules are specifically eluted. In a sequential purification procedure, the antibody is specifically bound to a first anti-immunoglobulin antibody, non-specifically bound molecules are removed in a wash step, and the specifically bound molecules are specifically eluted. The eluant from the first anti-immunoglobulin antibody is then specifically bound to a second anti-immunoglobulin antibody. The non-specifically bound molecules are removed in a wash step, and the specifically bound molecules are specifically eluted. In a preferred embodiment, the antibody is sequentially purified by a first and second anti-immunoglobulin antibody selected from the group consisting of antibodies which specifically bind heavy and light chain constant regions. In a more preferred embodiment, the antibody is sequentially purified by a first and second anti-immunoglobulin antibody selected from the group consisting of antibodies which specifically bind the heavy chain constant region of IgG and light chain constant regions of kappa and lambda. In an even more preferred embodiment, the anti-immunoglobulin antibody is selected from the group consisting of antibodies which specifically bind the light chain constant regions of kappa and lambda.

Diagnostic Methods

The present invention also describes a diagnostic system, preferably in kit form, for assaying for the presence of Streptococcus, in particular Streptococcus pneumoniae, in a biological sample where it is desirable to detect the presence, and preferably the amount, of bacteria in a sample according to the diagnostic methods described herein.

The diagnostic system includes, in an amount sufficient to perform at least one assay, a binding member composition according to the present invention, preferably as a separately packaged reagent, and more preferably also instruction for use.

The biological sample can be a tissue, tissue extract, fluid sample or body fluid sample, such as blood, plasma or serum.

Packaged refers to the use of a solid matrix or material such as glass, plastic (e.g., polyethylene, polypropylene or polycarbonate), paper, foil and the like capable of holding within fixed limits a binding member of the present invention. Thus, for example, a package can be a glass vial used to contain milligram quantities of a contemplated labeled binding member preparation, or it can be a microtiter plate well to which microgram quantities of a contemplated binding member has been operatively affixed, i.e., linked so as to be capable of binding a ligand.

"Instructions for use" typically include a tangible expression describing the reagent concentration or at least one assay method parameter such as the relative amounts of reagent and sample to be admixed, maintenance time periods for reagent/sample admixtures, temperature, buffer conditions and the like.

A diagnostic system of the present invention preferably also includes a label or indicating means capable of signaling the formation of a binding reaction complex containing a binding member complexed with the preselected ligand.

Any label or indicating means can be linked to or incorporated in an expressed polypeptide, or phage particle that is used in a diagnostic method. Such labels are themselves well-known in clinical diagnostic chemistry.

The labeling means can be a fluorescent labeling agent that chemically binds to antibodies or antigens without denaturing them to form a fluorochrome (dye) that is a useful immunofluorescent tracer. Suitable fluorescent labeling agents are fluorochromes such as fluorescein isocyanate (FIC), fluorescein isothiocyante (FITC), 5-dimethylamine-1-naphthalenesulfonyl chloride (DANSC), tetramethylrhodamine isothiocyanate (TRITC), lissamine, rhodamine 8200 sulphonyl chloride (RB 200 SC) and the like. A description of immunofluorescence analysis techniques is found in DeLuca, "Immunofluorescence Analysis", in Antibody As a Tool, Marchalonis, et al., eds., John Wiley & Sons, Ltd., pp. 189-231 (1982), which is incorporated herein by reference.

In preferred embodiments, the indicating group is an enzyme, such as horseradish peroxidase (HRP), glucose oxidase, or the like. In such cases where the principal indicating group is an enzyme such as HRP or glucose oxidase, additional reagents are required to visualize the fact that a receptor-ligand complex (immunoreactant) has formed. Such additional reagents for HRP include hydrogen peroxide and an oxidation dye precursor such as diaminobenzidine. An additional reagent useful with glucose oxidase is 2,2'-amino-di-(3-ethyl-benzthiazoline-G-sulfonic acid) (ABTS).

Radioactive elements are also useful labeling agents and are used illustratively herein. An exemplary radiolabeling agent is a radioactive element that produces gamma ray emissions. Elements which themselves emit gamma rays, such as ^{124}I, ^{125}I, ^{128}I, ^{132}I and ^{51}Cr represent one class of gamma ray emission-producing radioactive element indicating groups. Particularly preferred is ^{125}I. Another group of useful labeling means are those elements such as ^{11}C, ^{18}F, ^{15}O and ^{13}N which themselves emit positrons. The positrons so emitted produce gamma rays upon encounters with electrons present in the animal's body. Also useful is a beta emitter, such ^{111}indium of ^{3}H.

The linking of labels, i.e., labeling of, polypeptides and proteins or phage is well known in the art. For instance, proteins can be labeled by metabolic incorporation of radioisotope-containing amino acids provided as a component in the culture medium. See, for example, Galfre et al., Meth. Enzymol., 73:3-46 (1981). The techniques of protein conjugation or coupling through activated functional groups are particularly applicable. See, for example, Aurameas, et al., Scand. J. Immunol., Vol. 8 Suppl. 7:7-23 (1978), Rodwell et al., Biotech., 3:889-894 (1984), and U.S. Pat. No. 4,493,795.

The diagnostic systems can also include, preferably as a separate package, a specific binding agent. A "specific binding agent" is a molecular entity capable of selectively binding a binding member species of the present invention or a complex containing such a species, but is not itself a binding member of the present invention. Exemplary specific binding agents are antibody molecules, complement proteins or fragments thereof, S. aureus protein A, and the like. Preferably the specific binding agent binds the binding member species when that species is present as part of a complex.

In preferred embodiments, the specific binding agent is labeled. However, when the diagnostic system includes a specific binding agent that is not labeled, the agent is typically used as an amplifying means or reagent. In these embodiments, the labeled specific binding agent is capable of specifically binding the amplifying means when the amplifying means is bound to a reagent species-containing complex.

The diagnostic kits of the present invention can be used in an "ELISA" format to detect the quantity of a preselected ligand in a fluid sample. "ELISA" refers to an enzyme-linked immunosorbent assay that employs an antibody or antigen bound to a solid phase and an enzyme-antigen or enzyme-antibody conjugate to detect and quantify the amount of an antigen present in a sample and is readily applicable to the present methods.

Thus, in some embodiments, a binding member of the present invention can be affixed to a solid matrix to form a solid support that comprises a package in the subject diagnostic systems.

A reagent is typically affixed to a solid matrix by adsorption from an aqueous medium although other modes of affixation applicable to proteins and polypeptides can be used that are well known to those skilled in the art. Exemplary adsorption methods are described herein.

Useful solid matrices are also well known in the art. Such materials are water insoluble and include the cross-linked dextran available under the trademark SEPHADEX from Pharmacia Fine Chemicals (Piscataway, N.J.); agarose; beads of polystyrene beads about 1 micron to about 5 millimeters in diameter available from Abbott Laboratories of North Chicago, Ill.; polyvinyl chloride, polystyrene, cross-linked polyacrylamide, nitrocellulose- or nylon-based webs such as sheets, strips or paddles; or tubes, plates or the wells of a microtiter plate such as those made from polystyrene or polyvinylchloride.

The binding member species, labeled specific binding agent or amplifying reagent of any diagnostic system described herein can be provided in solution, as a liquid dispersion or as a substantially dry power, e.g., in lyophilized form. Where the indicat-

ing means is an enzyme, the enzyme's substrate can also be provided in a separate package of a system. A solid support such as the before-described microtiter plate and one or more buffers can also be included as separately packaged elements in this diagnostic assay system.

Diagnostic methods

The present invention also contemplates various assay methods for determining the presence, and preferably amount, of a Streptococcus, in particular Streptococcus pneumoniae, typically present in a biological sample.

Accordingly, the present invention relates to a method of detecting or diagnosing a disease or disorder associated with Pneumococcus in an individual comprising

- providing a biological sample from said invidual
- adding at least one binding member as defined above to said biological sample,
- detecting binding members bound to said biological sample, thereby detecting or diagnosing the disease or disorder.

The bound binding members may be detected either directly or indirectly, to the amount of the Streptococcus in the sample.

Those skilled in the art will understand that there are numerous well known clinical diagnostic chemistry procedures in which a binding reagent of this invention can be used to form an binding reaction product whose amount relates to the amount of the ligand in a sample. Thus, while exemplary assay methods are described herein, the invention is not so limited.

Various heterogenous and homogeneous protocols, either competitive or noncompetitive, can be employed in performing an assay method of this invention.

Binding conditions are those that maintain the ligand-binding activity of the receptor. Those conditions include a temperature range of about 4 to 50 degrees Centigrade, a pH value range of about 5 to 9 and an ionic strength varying from about that of distilled water to that of about one molar sodium chloride.

The detecting step can be directed, as is well known in the immunological arts, to either the complex or the binding reagent (the receptor component of the complex). Thus, a secondary binding reagent such as an antibody specific for the receptor may be utilized.

Alternatively, the complex may be detectable by virtue of having used a labeled receptor molecule, thereby making the complex labeled. Detection in this case comprises detecting the label present in the complex.

A further diagnostic method may utilize the multivalency of a binding member composition of one embodiment of this invention to cross-link ligand, thereby forming an aggregation of multiple ligands and polypeptides, producing a precipitable aggregate. This embodiment is comparable to the well known methods of immune precipitation. This embodiment comprises the steps of admixing a sample with a binding member composition of this invention to form a binding admixture under binding conditions, followed by a separation step to isolate the formed binding complexes. Typically, isolation is accomplished by centrifugation or filtration to remove the aggregate from the admixture. The presence of binding complexes indicates the presence of the preselected ligand to be detected.

Pharmaceutical compositions

In a preferred aspect the present invention contemplates pharmaceutical compositions useful for practicing the therapeutic methods described herein. Pharmaceutical compositions of the present invention contain a physiologically tolerable carrier together with at least one species of binding member as described herein, dissolved or dispersed therein as an active ingredient. In a preferred embodiment, the pharmaceutical composition is not immunogenic when administered to a human individual for therapeutic purposes, unless that purpose is to induce an immune response.

In one aspect the invention relates to a pharmaceutical composition comprising at least one binding member as defined above. In a preferred embodiment the pharmaceutical composition comprises at least two different binding members as defined above in order to increase the effect of the treatment.

As used herein, the terms "pharmaceutically acceptable", "physiologically tolerable" and grammatical variations thereof, as they refer to compositions, carriers, diluents and reagents, are used interchangeably and represent that the materials are capable of administration to or upon a human without the production of undesirable physiological effects such as nausea, dizziness, gastric upset and the like.

The preparation of a pharmacological composition that contains active ingredients dissolved or dispersed therein is well understood in the art. Typically such compositions are prepared as sterile injectables either as liquid solutions or suspensions, aqueous or non-aqueous, however, solid forms suitable for solution, or suspensions, in liquid prior to use can also be prepared. The preparation can also be emulsified.

The active ingredient can be mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient and in amounts suitable for use in the therapeutic methods described herein. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol or the like and combinations thereof. In addition, if desired, the composition can contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents and the like which enhance the effectiveness of the active ingredient.

The pharmaceutical composition of the present invention can include pharmaceutically acceptable salts of the components therein. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the polypeptide) that are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, tartaric, mandelic and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine and the like.

Physiologically tolerable carriers are well known in the art. Exemplary of liquid carriers are sterile aqueous solutions that contain no materials in addition to the active ingredients and water, or contain a buffer such as sodium phosphate at physiological pH value, physiological saline or both, such as phosphate-buffered saline. Still

further, aqueous carriers can contain more than one buffer salt, as well as salts such as sodium and potassium chlorides, dextrose, propylene glycol, polyethylene glycol and other solutes.

Liquid compositions can also contain liquid phases in addition to and to the exclusion of water. Exemplary of such additional liquid phases are glycerin, vegetable oils such as cottonseed oil, organic esters such as ethyl oleate, and water-oil emulsions.

A pharmaceutical composition contains a binding member of the present invention, typically an amount of at least 0.1 weight percent of antibody per weight of total pharmaceutical composition. A weight percent is a ratio by weight of antibody to total composition. Thus, for example, 0.1 weight percent is 0.1 grams of antibody per 100 grams of total composition.

The invention also relates to a method for preparing a medicament or pharmaceutical composition comprising an antibody of the invention, the medicament being used for immunotherapy of a disease or disorder associated with Streptococcus, in particular Streptococcus pneumoniae, such as pneumonia, meningitis and sepsis, comprising admixing at least one binding member as defined above with a physiologically acceptable carrier.

Furthermore, the invention relates to the use of a binding member as defined above for the production of a pharmaceutical composition for the treatment of a disease or disorder associated with Streptococcus, in particular Streptococcus pneumoniae, such as pneumonia, meningitis and sepsis.

The pharmaceutical composition may also be a kit-in-part further including an antibiotic agent, such as antibiotics selected from β-lactams, cephalosporins, penicilins and aminoglycosides, and/or include an immunostimulating agent, such as cytokines, interferons, growth factors, for example GCSF or GM-CSF. The kit-in-part may be used for simultaneous, sequential or separate administration.

Furthermore, the pharmaceutical composition may include the binding member according to the invention in combination with the Streptococcus protein PsaA, in particular as a vaccine. It has been found that by combining the binding member ac-

cording to the invention with the protein PsaA, the immunising properties of the combination product is better than for the protein PsaA alone. This may be due to the fact, that the protein PsaA is presented to the immune system by the binding member.

Therapeutic methods

The binding members according to the present invention are particular useful in therapeutic methods due to their high affinity and specificity. Accordingly, the binding members can be used immunotherapeutically towards a disease or disorder associated with Streptococcus, in particular Streptococcus pneumoniae, such as pneumonia, meningitis and sepsis.

The term "immunotherapeutically" or "immunotherapy" as used herein in conjunction with the binding members of the invention denotes both prophylactic as well as therapeutic administration. Thus, the binding members can be administered to high-risk patients in order to lessen the likelihood and/or severity of disease, administered to patients already evidencing active infection, or administered to patients at risk of infection.

The dosage ranges for the administration of the binding members of the invention are those large enough to produce the desired effect in which the symptoms of the disease are ameliorated or the likelihood of infection decreased. Generally, the dosage will vary with the age, condition, sex and extent of the disease in the patient and can be determined by one of skill in the art. The dosage can be adjusted by the individual physician in the event of any complication.

A therapeutically effective amount of an binding member of this invention is typically an amount of antibody such that when administered in a physiologically tolerable composition is sufficient to achieve a plasma concentration of from about 0.1 microgram (µg) per milliliter (ml) to about 100 µg/ml, preferably from about 1 µg/ml to about 5 µg/ml, and usually about 5 µg/ml. Stated differently, the dosage can vary from about 0.1 mg/kg to about 300 mg/kg, preferably from about 0.2 mg/kg to about 200 mg/kg, most preferably from about 0.5 mg/kg to about 20 mg/kg, in one or more dose administrations daily, for one or several days.

The binding members of the invention can be administered parenterally by injection or by gradual infusion over time. Although the infection may be systemic and therefore most often treated by intravenous administration of pharmaceutical compositions, other tissues and delivery means are contemplated where there is a likelihood that targeting a tissue will result in a lessening of the disease. Thus, antibodies of the invention can be administered parenterally, such as intravenously, intraperitoneally, intramuscularly, subcutaneously, intracavity, transdermally, and can be delivered by peristaltic means.

The pharmaceutical compositions containing a binding member of this invention are conventionally administered intravenously, as by injection of a unit dose, for example. The term "unit dose" when used in reference to a pharmaceutical composition of the present invention refers to physically discrete units suitable as unitary dosage for the subject, each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required diluent; i.e., carrier, or vehicle.

The therapeutic method may further include the use of a kit-in-part as defined above.

Examples

The invention is further explained through the examples below, the examples are not to be construed as limiting to the invention.

Example 1

Production of anti-PsaA antibodies

Mice having the ability of making fully human antibodies were used. The mice were HuMAb-Mouse, obtained from Medarex, Inc.

His6-psaA protein was used as antigen for the immunization experiments. The concentrations of these proteins were calculated by spectrophotometer, and their purity

was ascertained by SDS-PAGE after silver staining. Proteins were prepared for immunization using complete Freund's, incomplete Freund's, and RIBI as an adjuvant, as appropriate. Groups of 12 to 18 male HuMAb mice were used for all immunization experiments. Each mouse was immunized by intraperitoneal (i.p.) and subcutaneous injection of 20-50 µg of His6-psaA (PsaA amino acid sequence is shown in Figure 15) at three-week intervals. Some mice received 5x10e8 heat-inactivated Streptococcus pneumoniae R6 cells i.p. Sera were collected from mice by retro-orbital bleeding. Titers to rpsaA were determined by ELISA. Mice were boosted intravenously prior to sacrifice.

8 fusions were performed and resulted in 65 viable cell clones. These were subcloned and 42 positive clones identified by the method described in 1a below were selected for further research. Among these 26 were selected and monoclonal antibodies purified. 3 were discarded because of poor reactivity, thus 23 clones were evaluated. Among the 23 clones a prioritized list of candidate MAbs was established. The four top-prioritized were further evaluated in an in vivo model.

1a) Identification of Streptococcus pneumoniae R6 binding antibodies

Hydridoma supernatants are tested in 3 dilutions against *S.pneumococus* R6 in a sandwich enzyme linked immunosorbent assay with reagent excess.

Devices:

ELISA reader, BIO-TEK EL 800
ELISA washer, handheld model
Incubator 37 °C
Pipettes

Materials:

Tips
Reagent tray
Plate cover
96-well microtiterplate (Nunc Maxisorp)

Bacteria:

S.pneumococcus R6 (SSI, Pneumokoklaboratoriet) from frozen stock with a concentration of 10e10/ml. Thaw rapidly at 37°C and dilute 1:100 in coating buffer.

Reagents:

Na2CO3, pH 9.6
PBS, pH 7.4
Tween 20,
Rabbit-anti-Human IgA,G,M HRP-conjugated, DAKO #P212
Rabbit-anti-Mouse Ig HRP-conjugated, DAKO #P260
Swine-anti-Rabbit Ig HRP-conjugated, DAKO #P217
0.1 M Citric Acid, pH 5.0
OPD 10 mg tablets, Kem-En-Tec no. 4260
H2O2
H2SO4

Coating Buffer: Na2CO3 0.05 M, pH 9.6, expire two weeks after production, storage at 4°C.

Dilution and wash buffer: PBS with 0.1% Tween20, expire one week after production, storage at 4°C

Secondary Antibody: Dilute 1:1500 in dilution buffer, freshly made every day.

Substrate: Dissolve 1 OPD tablet in 10 ml Citric Acid and add 10 µl H2O2. Freshly made every day.

Stop reagent: 1.2 M H2SO4, expire 1 year after production, storage at RT.

Controls:

Blank: PBS with 0.1% Tween 20

Positive controls: Affinity purified Ra-a-PsaA

Samples:

Hybridoma culture supernatants diluted 1:2, 1:50 and 1:1250 in dilution buffer.

Procedure:

100 µL bacteria per well (=10e7) are coated into 96-microwells and the plate is incubated overnight at 4°C. Discard excess fluid. The plate is washed three times with 300 µL wash buffer and can hereafter be stored at 4°C for a period of 1 month.

100 µL blank, positive control or sample is added and the plate is incubated 2 hours at 37°C. The plate is washed three times with 300 µL wash buffer
100 µL secondary antibody is added and the plate is incubated 1 hour at 37°C.
The plate is washed three times with 300 µL wash buffer. The plate is washed two times with 300 µL Dem. H2O.100 µL substrate is added and the plate is incubated 30 min at RT. 100 µL stop reagent is added and the plate is read at A490 nm.

Example 2

In vitro effect of monoclonal anti-PsaA antibodies

The monoclonal antibodies obtained in Example 1 were tested in vitro against Pneumococcus of various strains identifying antibodies against whole bacteria (ELISA) see method 1, and using Western blot, see method 2, below.

Method 2

Identification of bacteria binding antibodies – Western blot.

First bacterial proteins are transferred from NuPAGE Tris-Bis Gels to polyvinylidene difluoride (PVDF) membranes by the following method.

Samples:
Electrophoresed NuPAGE Tris-Bis Gels
Devices:
XCell Surelock™ Mini-Cell with Blot Module
Power supply
Materials:
Pre-cut blotting PVDF membrane (0.45 µm) and filter papers (Invitrogen #LC2005)
Pipettes
Tips
Trays
Reagents:
NuPAGE Transfer Buffer (Invitrogen #NP0006)

Milli-Q grade H_2O
Ethanol, 96%

Procedure:

Transferring One Gel

a) 500 ml of Transfer Buffer was prepared by adding 25 ml 20X NuPAGE® Transfer Buffer and 100 ml Ethanol 96% to 375 ml Milli-Q grade H_2O. Blotting pads were soaked in 350 ml of Transfer Buffer. PVDF membrane was soaked in 96% Ethanol for 30 sec. and wash in transfer buffer for 2 min, and the filter paper was briefly soaked in Transfer Buffer.

A piece of pre-soaked filter paper was placed on top of the gel (adhered to the bottom plate) and any trapped air bubbles were removed.

The plate was turned over so the gel and filter paper were facing downwards over a gloved hand or clean flat surface. The pre-soaked transfer membrane was placed on the gel and trapped air bubbles removed.

Another pre-soaked filter paper was placed on top of the membrane and any air bubbles were removed.

b) Two soaked blotting pads ware placed into the cathode (-) core of the blot module. The gel/membrane assembly was carefully picked up and placed on blotting pad in the correct orientation, so the gel is closest to the cathode core. Two soaked blotting pads are placed on top of the sandwich, and the anode (+) core on top of the pads.

c) The blot module was held together firmly and slid it into the guide rails on the Lower Buffer Chamber. The Gel Tension Wedge was sledged into the Lower Buffer Chamber and the Wedge locked into position.

The blot module was filled with Transfer Buffer until the gel/membrane assembly was covered. The Outer Buffer Chamber was filled with with 650 ml Milli-Q grade H_2O, the lid placed on the unit and the electrical leads connected to the power sup-

ply. Transfer for PVDF membranes were performed using 30 V constant for 1 hour. The expected start current was 170 mA and end current is 110 mA.

Western Blots were developed, and the dried membranes were stored in closed container/pouch at 4°C for 1 week

Transferring Two Gels

1. Repeat Steps a) above twice to prepare 2 gel/membrane sandwiches.
2. Place two pre-soaked blotting pads on the cathode core of the blot module.
3. Place the first gel/membrane assembly on the blotting pad in correct orientation, so the gel is closest the cathode core.
4. Add another pre-soaked blotting pad on top of the first membrane assembly.
5. Place the second gel/membrane sandwich on top of the blotting pad in the correct orientation so the gel is closest the cathode core.
6. Proceed with Steps c) from Transferring One Gel.

The identification is conducted as described below:

PVDF membranes with electrophoretically transferred proteins, bacterial lysate(s) or lipopolysaccharide.

Materials:

Tubes

Trays

Pipettes

Tips

Reagents:

WesternBreeze Blocker/Diluent A+B, Invitrogen no. WB7050

Western Breeze Wash Solution (16x), Invitrogen no. WB7003 .

NBT/BCIP Liquid Substrate, Sigma-Aldrich no. B3679

Simply Blue Safestain, Invitrogen no. LC 6060

Rabbit anti-Human IgG AP, DAKO D0336

Rabbit anti-Mouse Ig AP, DAKO D0314

Swine anti-Rabbit Ig AP, DAKO D0306

Milli-Q grade H2O

Ethanol 96%

Wash buffer:

10 ml Western Breeze Wash Solution (16x)

150 ml Milli-Q grade H2O
Blocking Solution:
5 ml Milli-Q grade H2O
2 ml Blocker/Diluent (Part A)
3 ml Blocker/Diluent (Part B)
Primary Antibody Solution:
7 ml Milli-Q grade H2O
2 ml Blocker/Diluent (Part A)
1 ml Blocker/Diluent (Part B)
Primary antibody to final concentration 0.2-1 µg/ml
Secondary Antibody Solution:
7 ml Milli-Q grade H2O
2 ml Blocker/Diluent (Part A)
1 ml Blocker/Diluent (Part B)
5 µl Secondary antibody (Final dilution 1:2000)

Procedure:

1. Dried PVDF membranes are re-wetted in Ethanol 96% and rinsed two times for 5 min each in 20 ml of Milli-Q grade H2O, proceed to step 4.
2. Freshly blotted membranes are placed on glass plate and MW marker is cut off. MW marker is stained for 15-30 min in Simply Blue SafeStain and de-stained with 30% Ethanol until background is clear. Wash twice in Milli-Q grade H2O.
3. The membrane is washed two times for 5 min each in 20 ml Milli-Q grade H2O.
4. Place membrane in 10 ml of Blocking Solution in covered, plastic dish for 30 min at RT, gently shaking. Decant Blocking Solution
5. Rinse with 20 ml of H2O for 5 min at RT, gently shaking. Decant. Repeat once. Membrane can now be cut into strips if necessary.
6. Incubate membrane with 10 ml of Primary Antibody Solution for 1 hour at RT, gently shaking. Alternatively, incubate o.n. at 4°C.
7. Wash four times for 5 min each time with 20 ml Wash Buffer.
8. Incubate membrane with 10 ml of Secondary Antibody Solution for 30 min at RT.
9. Wash four times for 5 min each time with 20 ml Wash buffer.
10. Rinse the membrane three times for 2 min each time with 20 ml of Milli-Q grade H2O.
11. Incubate membrane in 5 ml of NBT/BCIP Liquid Substrate until purple bands

develop (1-60 min.).

12. Stop development by rinsing the membrane three times for 2 min each time with 20 ml of Milli-Q grade H2O.

13. Dry the membrane on a clean piece of filter paper.

Results

In Figure 20, the results from the in vitro tests are listed.

Example 3

Measurement of anti-PsaA antibody affinity

The following general description was applied in all affinity measurements.

Instrument and software used:

a. BIAcore 3000, surface plasmon resonance instrument
b. BiaEval v3.2, software for data analysis.
c. All running buffers (HBS-EP/HBS-P) from Biacore.

Method to immobilize Protein G via amines on a CM5 chip (Amine coupling method)

a. Normalize the chip at least twice with appropriate buffer
b. A 0.5 µg/mL dilution of Protein-G is made in 10mM sodium acetate buffer of pH 2.9
c. Activate the CM5 chip for 7 minutes, by flowing freshly mixed EDC & NHS at a flow rate of 5 µL/min, according to the method mentioned in Biacore Handbook.
d. Inject Protein-G sample for 22 minutes over this activated surface.
e. Deactivate by flowing 1M ethonolamine-HCl for 10 minutes.
f. This methods couples about 10000 RUs of Protein-G on the activated surface.

g. For the blank surface, the activation and deactivation same procedure is followed without the injection of Protein-G.

Method to immobilize PsaA via amines on a CM5 chip (Amine coupling method)

a. A CM5 chip was normalized as above
b. PsaA dilutions are made in concentrations ranging from 50 to 150 µg/mL, in sodium 10mM acetate buffer of pH 4.0
c. The chip is activated for 7 minutes by flowing freshly mixed EDC and NHS.
d. Inject PsaA, made in acetate buffer, by manual injection until the amount captured on the chip reaches the desired level (in our case, 350 and 800 RUs)
e. Deactivate the chip by injecting 1M ethanolamine-HCl for 10 minutes
f. Regenerate the chip with a mild acid or base to remove unbound/loosely bound molecules from the chip surface.
g. The blank surface is generated in the same method, but without the step of injecting the protein.

Method to determine the avidity of binding of anti-PsaA antibodies with PsaA

a. The general methodology followed to measure avidity of antibodies is to flow antibodies of at least five different concentrations over the antigen surface (described above) at high flow rates. The high flow rates are required to minimize the antibody re-binding to the antigen surface. The association and dissociation phases could vary between 5 to 10 minutes and 30 to 40 minutes respectively. Though this experimental design will lead to the measurement of avidities of the antibodies, we try to minimize the effect of this phenomena in the estimation of avidity. The data analysis was carried out after carefully not including regions that exhibited biphasic behavior of association /dissociation, which was then fit to a 1:1 Langmuir model. The method of selecting the data for analysis ensures that the estimates of avidity are closer to the true affinities.

b. Antibody concentration: 3,2,1,0.75 or 0.5 µg/mL (corresponding to 40.02, 26.68, 13.34, 10.0 & 6.67 nM of *binding sites*). All dilutions were made in the running buffer, HBS-EP, pH 7.2.

c. Flow rate of 30 µL/min. Association phase 8min, dissociation phase 30 to 40 min.

d. Regeneration of the surface: flow rate 100 µL/min, buffer: 100mM HCl w/150mM NaCl., time: 1-2 minutes.

Experiment 3a) Binding affinity of 8 purified anti-PsaA huMabs

Reagents:

Name	Conc.	Lot #
PsaA	0.6mg/mL	021127
11H10	1.24mg/mL	
12E10	1.35mg/mL	
9C3	0.83mg/mL	
8A12	1.33mg/mL	
4F10	1.85mg/mL	
7H7	1.09mg/mL	
7D12	1.61mg/mL	
6D10	0.80mg/mL	

Instrument Used: Biacore: 3000
Chip used: Coupling Buffer: 10mM Acetate, pH 4.0
Coupling Conc: 50-150 ug/mL
Amt. immobilized (02/12/03):
Fc1 & 3= Blank Fc2 = PsaA: 353RUs Fc4: PsaA: 824RUs
Amt. immobilized (02/20/03):
Fc1 & 3= Blank Fc2 = PsaA: 1145RUs Fc4: PsaA: 355RUs

Experimental Conditions:

Antibody Conc.: 5, 4, 3, 2 & 1ug/mL (66.7, 53.36, 40.02, 26.68 &13.34nM)
Running buffer: HBS-EP Flow rate: 30uL/min
Association Time: 8 min. Dissociation Time: 40min
Regeneration Buffer: 100mM HCl+150mMNaCl
Flow rate: 100uL/min Regeneration Time: 1 min

Results:

Sample ID	$K_D x10^{-9}$ (M)	$k_a x10^4$ (1/Ms)	$k_d x10^{-5}$ (1/s)
11H10	2.45	6.86	16.8
12E10	0.64	7.93	5.07
9C3*	7.07	1.02	7.2
8A12	Negligible binding at the high density PsaA surface		
4F10	1.33	7.67	10.2
7H7	1.14	6.32	7.18
7D12	Poor binding at both the low & high density surface		
6D10	0.85	13.5	11.5

*Binding curves at higher conc. did not reach the saturation.

Experiment 3b) Binding affinity of 3 purified anti-PsaA HuMabs

Reagents:

Name	Conc.	Lot #
PsaA	0.36mg/mL	064014B
9A7	3.83 mg/mL	
1G9	0.98mg/mL	
15E5	1.36 mg/mL	

Instrument Used: Biacore: 3000

Chip used: CM5

Coupling Buffer: 10mM Acetate, pH 4.0 Coupling Conc: 50-150 ug/mL

Amt. immobilized:

Fc1 & 3= Blank Fc2 = PsaA: 353 RUs Fc4: PsaA: 824 RUs

Experimental Conditions:

Antibody Conc.: 4, 3, 2, 1 & 0.5ug/mL (53.36, 40.02, 26.68, 13.34 & 6.67nM)

Running buffer: HBS-EP Flow rate: 25uL/min

Association Time: 5 min. Dissociation Time: 45min

Regeneration Buffer: 100mM HCl+150mM NaCl

Flow rate: 100uL/min Regeneration Time: 1 min

Results:

Sample ID	K_D (M)	k_a (1/Ms)	k_d (1/s)
1G9	6.34×10^{-11}	4.35×10^{5}	2.87×10^{-5}
9A7	3.74×10^{-11}	3.77×10^{5}	1.41×10^{-5}
15E5	1.18×10^{-10}	5.71×10^{4}	6.72×10^{-6}

Experiment 3c) Binding affinity of 14 purified anti-PsaA HuMabs

Reagents:

Name	Conc.	Lot #
PsaA	0.6mg/mL	021127
3D10	3.5mg/mL	
4B11	3.93mg/mL	
7E8	4.42mg/mL	
7H8	3.18mg/mL	
10G6	2.36mg/mL	
2G6	1.94mg/mL	

2G8	5.35mg/mL
9C7	3.35mg/mL
10G9	1.07mg/mL
5E10	3.21mg/mL
7A4	2.23mg/mL
10E5	2.07mg/mL
7F12	0.256mg/mL
9E2	0.219mg/mL

Instrument Used: Biacore: 3000

Chip used: CM5 Chip prepared on: 02/12/03

Coupling Buffer: 10mM Acetate, pH 4.0 Coupling Conc: 50-150 ug/mL

Amt. immobilized (02/12/03):

Fc1 & 3= Blank Fc2 = PsaA: 353RUs Fc4: PsaA: 824RUs

Experimental Conditions:

Antibody Conc.: 3, 2, 1, 0.75 & 0.50ug/mL (40.02, 26.68, 13.34, 10.0, 6.67nM)

Running buffer: HBS-EP Flow rate: 30uL/min

Association Time: 8 min. Dissociation Time: 30-40min

Regeneration Buffer: 100mM HCl+150mMNaCl

Flow rate: 100uL/min Regeneration Time: 45sec-1 min

Results:

Sample ID	K_D x10^{-10} (M)	k_a x10^5 (1/Ms)	k_d x10^{-5} (1/s)
3D10	1.26	2.25	2.83
4B11	0.56	1.45	0.82
7E8*	0.78	0.66	0.51
7H8	1.71	1.65	2.82
10G6†	Negligible binding at high density PsaA surface		
2G6	1.75	2.07	3.61

2G8*	1.16	0.79	0.92
9C7†	0.82	0.21	0.17
10G9*	3.14	0.35	1.1
5E10*	1.61	0.70	0.11
7A4	2.12	1.64	3.49
10E5	0.39	2.2	0.86
7F12	0.47	5.67	2.67
9E2	0.42	7.33	3.1

*Binding curves at higher conc. did not reach the saturation.

†Needs to repeat the exp. at higher conc. of the mab

Example 4

In vivo testing of candidate antibodies

The effect of anti-PsaA huMabs in the treatment of infection caused by Pneumococcus was determined as described below.

Materials:

- Transgenic (human CD64) female mice (8 – 12 weeks, weight 19-20 g))
- 0,9% saline (MU)
- PBS pH 7,0
- 5% blood plates
- Filtered bovine broth
- Monoclonal antibodies:
  - anti-PsaA 5-9A7
  - anti-PsaA 1-15E5
  - anti-PsaA 7-1G9
  - anti-PsaA 4-3D10

Strains: Pneumococcus D39 (type 2) *(F1/S1/Æ2)*

Method

Day –1:

Pn.-strain is seeded on a 4 x 5% bloodplate, and incubated overnight at 35°C.

Day 0

The Pneumococcus strain is suspended in filtered broth to 10^8 CFU/ml (jf. MU/F074-01), and diluted to 1x 10^6 CFU/ml (50 µl 10^8 CFU/ml i 4.95 ml PBS) and further diluted with antibody (see scheme). The bacteria/antibody mixture is shaken and incubated for 10 min. at 35° and then the mice are inoculated with 0.5 ml i.p.

Solution	**Final conc.**	**ANTIBODY** (500 µg/ml)	**Bacteria. appr. 10^6 CFU/ml**	**PBS**
Cage 1	10^5 CFU/ml	none	0.6 ml	5.40 ml
Cage 2	200 µg/10^5 CFU/ ml	1.6 ml	0.4 ml	2.00 ml
Cage 3	20 µg/10^5 CFU/ ml	0.2 ml	0.5 ml	4.30 ml
Cage 4	2 µg/10^5 CFU/ ml	0.02 ml	0.5 ml	4.48 ml

Scheme

Cage	No. mice	Antibody/bact./mice	Mice # withdrawn time 2 hours	Mice # withdrawn time 5 hours
1	6-9	PBS/ 5x10^4 CFU/mice	1-2-3	4 – 9
2	6-9	40-100 µg/5x10^4 CFU/mice		10 – 15
3	6-9	10 µg/5x10^4 CFU/mice		16 – 21
4	6-9	1 µg/5x10^4 CFU/mice		22 – 27

*) Serum and peritoneal fluid was frozen at -20°C for antibody concentration measurements

Withdrawal of blood samples

The mice were sedated with CO_2. A cut was made in the axilla, and blood collected in tube glass 0. 100 µl was transferred to glass 1 and mixed thoroughly, whereafter 100 µl was spread on a bloodplate with a glass rod. Then CFU determination was conducted. The rest of the blood was centrifugated at 2000 x G for 7 min. and serum transferred to another tube, stored at –20°C.

Withdrawal of peritoneal fluid :

The mice were sacrificed, 2 ml sterile saline was injected into the abdomen, and 10 sec. later the fluid was withdrawn with a sterile Pasteur pipette and transferred to an Eppendorf tube. CFU determination was conducted.
The rest of the peritoneal fluid was stored at –20°C.

Results

The results are shown in Fig. 19A-E.

Example 5

Generation of anti-CD64 x anti-PsaA 5-9A7 Bispecific Antibody

$F(ab')_2$ fragments of each of the HuMAbs, anti-CD64 (88.53), and anti-PsaA 5-9A7 were generated by pepsin digestion and purified to homogeneity by Superdex 200 gel filtration chromatography. Size exclusion HPLC was performed and profiles are depicted for each of the $F(ab')_2$ in Figure 2. By this type of analysis both of the $F(ab')_2$ fragments were >95% pure.

A Fab' fragment of the 88.53 was generated by mild reduction of the inter-heavy chain disulfide bonds of the $F(ab')_2$ fragment with mercaptoethanolamine (MEA). The exact reducing conditions were determined prior to conjugation in small-scale experiments. Size exclusion HPLC was performed and the profile is depicted for the Fab'in Figure 3. By this type of analysis the 88.53 Fab' was >90% pure.

The Fab' fragment of the 88.53 was separated from free MEA by G-25 column chromatography. The Fab' fragment was incubated with dinitrothiobenzoate (DTNB) to generate a Fab-TNB conjugate.

A Fab' fragment of the 5-9A7 was generated by mild reduction of the inter-heavy chain disulfide bonds of the $F(ab')_2$ fragment with mercaptoethanolamine (MEA). The exact reducing conditions were determined prior to conjugation in small-scale experiments. Size exclusion HPLC was performed and the profile is depicted for the Fab'in Figure 4. By this type of analysis the 5-9A7 Fab' was >90% pure.

The Fab' fragment of the 5-9A7 was separated from free MEA by G-25 column chromatography and mixed with 88.53 Fab-TNB at a 1:1 molar ratio overnight at room temperature. The HPLC profile depicted in Figure 5 represents a profile of the conjugation mixture after 18 hours of incubation and before purification. This profile shows a mixture of bispecific antibody as well as unconjugated Fab' molecules.

The bispecifc antibody was purified from contaminating Fab' molecules by Superdex 200 size exclusion chromatography and the purified molecule was analyzed by HPLC. As shown in Figure 6 the 88.53 x 5-9A7 bispecific antibody was purified to near homogeneity.

For control anti-CD64 x anti-CD89 Bispecific Antibody were generated. $F(ab')_2$ fragments of each of the HuMAbs, anti-CD64 (88.53), and anti-CD89 (14A8) were generated by pepsin digestion and purified to homogeneity by Superdex 200 gel filtration chromatography. Size exclusion HPLC was performed and profiles are depicted for each of the $F(ab')_2$ in Figure 7. By this type of analysis both of the $F(ab')_2$ fragments were >95% pure.

A Fab' fragment of the 88.53 was generated by mild reduction of the inter-heavy chain disulfide bonds of the $F(ab')_2$ fragment with mercaptoethanolamine (MEA). The exact reducing conditions were determined prior to conjugation in small-scale experiments. Size exclusion HPLC was performed and the profile is depicted for the Fab'in Figure 8. By this type of analysis the 88.53 Fab' was >90% pure.

The Fab' fragment of the 88.53 was separated from free MEA by G-25 column chromatography. The Fab' fragment was incubated with dinitrothiobenzoate (DTNB) 16a and 16b to generate a Fab-TNB conjugate.

A Fab' fragment of the 14A8 was generated by mild reduction of the inter-heavy chain disulfide bonds of the $F(ab')_2$ fragment with mercaptoethanolamine (MEA). The exact reducing conditions were determined prior to conjugation in small-scale experiments. Size exclusion HPLC was performed and the profile is depicted for the Fab'in Figure 9. By this type of analysis the 14A8 Fab' was >95% pure.

The Fab' fragment of the 14A8 was separated from free MEA by G-25 column chromatography and mixed with 88.53 Fab-TNB at a 1:1 molar ratio overnight at room temperature. The HPLC profile depicted in Figure 10 represents a profile of the conjugation mixture after 18 hours of incubation and before purification. This profile shows a mixture of bispecific antibody as well as unconjugated Fab' molecules.

The bispecific antibody was purified from contaminating Fab' molecules by Superdex 200 size exclusion chromatography and the purified molecule was analyzed by HPLC. As shown in Figure 11 the 88.53 x 14A8 bispecific antibody was purified to near homogeneity.

<u>Characterization of the Binding Specificity of the anti-CD64 x anti-PsaA Bispecific Antibody – Bispecific ELISA</u>

1. ELISA plates were coated recombinant PsaA, 50 µl/well, 5 µg/ml and incubated overnight at 4°C.
2. The plates were blocked with 5% BSA in PBS.
3. Titrations of the bispecific antibody were added to the plate. Controls included the anti-CD64 x anti-CD89 bispecific (control bispecific) and the $F(ab')_2$ fragments of the anti-CD64 Ab, 88.53 or of the anti-PsaA Ab, 5-9A7.
4. The plates were then incubated with a supernatant containing a fusion protein consisting of soluble CD64 linked to the Fc portion of human IgM.

5. The plates were finally incubated with an alkaline phosphatase labeled goat anti-human IgM antibody. Positive wells were detected with the alkaline phosphatase substrate.

The anti-PsaA x anti-CD64 bispecific showed dose-dependent binding in this assay (see Figure 13). The control bispecific was not detected since it does not bind the PsaA, the anti-CD64 $F(ab')_2$ was not detected since it binds CD64 but not PsaA, and the anti-PsaA $F(ab')_2$ was not detected since it binds PsaA but not the soluble CD64-IgM fusion protein.

Characterization of the Binding Specificity of the anti-CD64 x anti-PsaA Bispecific Antibody – Binding to CD64 on Human CD64-transgenic Mice

Blood was taken from CD64 transgenic mice or from non-transgenic littermates, and was incubated with the 88.53 x 5-9A7 bispecific antibody at a concentration of 30 µg/ml for 30 minutes at room temperature.

The blood was washed and then incubated with an FITC-labeled anti-human IgG antibody for 30 minutes at room temperature. The red blood cells were lysed and the remaining leukocytes were analyzed for staining by flow cytometry. Regions corresponding to the lymphocyte, monocyte, and neutrophil populations were gated and analyzed separately. The results of the binding assay are depicted in Figure 14 (A-C). The black lines represent staining of cells from CD64 transgenic mice, the green lines cell from non-transgenic littermates.

Human CD64 is expressed on monocytes and, to a lesser extent, neutrophils of CD64 transgenic mice. As in humans, CD64 is not expressed by lymphocytes of the transgenic mice. The data in Figure 14 show that the bispecific antibody binds to CD64 transgenic monocytes and neutrohils but not to any cell populations derived from non-transgenic mice.

In summary, two bispecific antibodies, anti-CD64 x anti-PsaA and anti-CD64 x anti-CD89, were generated and purified to homogeneity. The 88.53 x 5-9A7 bispecific antibody was shown to bind simultaneously to CD64 and to PsaA. In addition this bispecific antibody binds to CD64 expressed by human CD64 transgenic mice.

Example 6

Sequencing of antibodies

Antibodies shown in the affinity table in Example 3b were sequenced by conventional methods, and the variable regions of the antibodies are shown in the Figures.

9A7	Fig. 16a and 16b
1G9	Fig. 17a and 17b
15E5	Fig. 18a and 18b

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Claims:

1. An isolated binding member comprising at least one binding domain capable of specifically binding Streptococcus pneumoniae surface adhesin A (PsaA) protein, said binding domain having a dissociation constant K_d for PsaA which is less than 1×10^{-6}M.

2. The isolated binding member according to claim 1, wherein the isolated binding member is a pure isolated binding member.

3. The isolated binding member according to claim 1, wherein the binding member is selected from antibodies or immunologically active fragments of antibodies or single chain of antibodies.

4. The isolated binding member according to claim 3, wherein the antibodies are selected from monoclonal antibodies, polyclonal antibodies or mixtures of monoclonal antibodies.

5. The isolated binding member according to claim 1, wherein the binding member is monospecific towards the PsaA protein.

6. The isolated binding member according to claim 1, wherein the binding member is bispecific having at least one portion specific towards the PsaA protein.

7. The isolated binding member according to claim 1, wherein the binding member is multispecific having at least one portion towards the PsaA protein.

8. The isolated binding member according to claim 1, wherein the binding domain is carried by a human antibody framework.

9. The isolated binding member according to claim 1, wherein the binding domain is carried by a humanised antibody framework.

10. The isolated binding member according to claim 1, wherein the binding domain comprises an amino acid sequence selected from SEQ ID NO: 1, from SEQ ID NO 2, from SEQ ID NO 3, and from SEQ ID NO 4 or a homologue thereof.

11. The isolated binding member according to claim 10, wherein the binding domain comprises at least two amino acid sequences selected from SEQ ID NO: 1, from SEQ ID NO 2, from SEQ ID NO 3, and from SEQ ID NO 4 or a homologue thereof.

12. The isolated binding member according to claim 10, wherein the binding domain comprises at least SEQ ID NO 2, and SEQ ID NO 3, or a homologue thereof.

13. The isolated binding member according to claim 10, wherein the binding domain comprises SEQ ID NO: 1, SEQ ID NO 2, and SEQ ID NO 3, or a homologue thereof.

14. The isolated binding member according to claim 10, wherein the binding domain comprises SEQ ID NO: 4, or a homologue thereof.

15. The isolated binding member according to claim 1, wherein the binding member is capable of binding PsaA from two or more different Pneumococcus serotypes.

16. The isolated binding member according to claim 10, wherein the homologue is at least 60 % homologous to one or more of the sequences selected from SEQ ID NO: 1, from SEQ ID NO 2, from SEQ ID NO 3, and from SEQ ID NO 4, such as at least 65 % homologous such as at least 70 % homologous, such as at least 75 % homologous, such as at least 80 % homologous, such as at least 85 % homologous, such as at least 90 % homologous, such as at least 95 % homologous, such as at least 98 % homologous.

17. The isolated binding member according to claim 1, wherein the dissociation constant is less than 5×10^{-9} M, such as less than 1×10^{-9} M.

18. The isolated binding member according to claim 10, wherein the binding domain is located in a V_L domain.

19. The isolated binding member according to claim 10, wherein the binding domain is located in a V_H domain.

20. The isolated binding member according to claim 10, wherein the binding domain is arranged as a complementarity-determining region (CDR) in the binding member.

21. The isolated binding member according to claim 1, wherein the fragment of antibodies are selected from Fab, Fab', $F(ab)_2$ and Fv.

22. The binding member accpording to any of the preceding claims, comprising at least a first binding domain and a second binding domain, said first binding domain being capable of specifically binding Streptococcus pneumoniae surface adhesin A (PsaA) protein, and said second binding domain is different from said first binding domain.

23. The isolated binding member according to claim 23, wherein the second binding domain is capable of specifically binding a mammalian protein, such as a human protein, such as a protein selected from CD64 or CD89.

24. The isolated binding member according to claim 24, wherein second binding domain is capable of specifically binding a mammalian cell, such as a human cell, such as a cell selected from a leucocyte, macrophages, lymphocytes, neutrophilic cells, basophilic cells, and eosinophilic cells.

25. The isolated binding member according to claim 23, second binding domain is capable of specifically binding a Pneumococcus protein.

26. The isolated binding member according to claim 26, wherein second binding domain is capable of specifically binding a PsaA epitope different from the first binding domain.

27. The isolated binding member according to claim 23, wherein the binding member comprises two binding domains.

28. The isolated binding member according to claim 28, wherein the two binding members are linked through a spacer region.

29. An isolated binding member comprising at least a first binding domain and a second binding domain, said first binding domain being capable of specifically binding Streptococcus pneumoniae surface adhesin A (PsaA) protein, and said second binding domain is different from said first binding domain.

30. The isolated binding member according to claim 29, wherein the isolated binding member is a pure isolated binding member.

31. The isolated binding member according to claim 29, wherein the binding member is selected from antibodies or immunologically active fragments of antibodies or single chain of antibodies.

32. The isolated binding member according to claim 31, wherein the antibodies are selected from monoclonal antibodies, polyclonal antibodies or mixtures of monoclonal antibodies.

33. The isolated binding member according to claim 29, wherein the binding member is monospecific towards the PsaA protein.

34. The isolated binding member according to claim 29, wherein the binding member is bispecific having at least one portion specific towards the PsaA protein.

35. The isolated binding member according to claim 29, wherein the binding member is multispecific having at least one portion towards the PsaA protein.

36. The isolated binding member according to claim 29, wherein the binding domain is carried by a human antibody framework.

37. The isolated binding member according to claim 29, wherein the binding domain is carried by a humanised antibody framework.

38. The isolated binding member according to claim 29, wherein the first binding domain comprises at least one of the amino acid sequence sets selected from the group of

the amino acid sequence sets SEQ ID NO 1 or a homologue thereof, SEQ ID NO 2 or a homologue thereof, and SEQ ID NO 3 or a homologue thereof, or the amino acid sequence sets SEQ ID NO 5 or a homologue thereof, SEQ ID NO 6 or a homologue thereof, and SEQ ID NO 7 or a homologue thereof, or the amino acid sequence sets SEQ ID NO 9 or a homologue thereof, SEQ ID NO 10 or a homologue thereof, and SEQ ID NO 11 or a homologue thereof, or the amino acid sequence sets SEQ ID NO 13 or a homologue thereof, SEQ ID NO 14 or a homologue thereof, and SEQ ID NO 15 or a homologue thereof, or the amino acid sequence sets SEQ ID NO 17 or a homologue thereof, SEQ ID NO 18 or a homologue thereof, and SEQ ID NO 19 or a homologue thereof, or the amino acid sequence sets SEQ ID NO 21 or a homologue thereof, SEQ ID NO 22 or a homologue thereof, and SEQ ID NO 23 or a homologue thereof.

39. The isolated binding member according to claim 38, wherein the first binding domain comprises at least one amino acid sequences selected from SEQ ID NO: 4, SEQ ID NO 8, SEQ ID NO 12, SEQ ID NO 16, SEQ ID NO 20, SEQ ID NO 24 or a homologue thereof.

40. The isolated binding member according to claim 38, wherein the first binding domain comprises the amino acid sequence sets SEQ ID NO 1 or a homologue thereof, SEQ ID NO 2 or a homologue thereof, and SEQ ID NO 3 or a homologue thereof, and the amino acid sequence sets SEQ ID NO 5 or a homologue thereof, SEQ ID NO 6 or a homologue thereof, and SEQ ID NO 7 or a homologue thereof.

41. The isolated binding member according to claim 38, wherein the first binding domain comprises the amino acid sequence sets SEQ ID NO 9 or a homologue thereof, SEQ ID NO 10 or a homologue thereof, and SEQ ID NO 11 or a homologue thereof, and the amino acid sequence sets SEQ ID NO 13 or a homologue thereof, SEQ ID NO 14 or a homologue thereof, and SEQ ID NO 15 or a homologue thereof.

42. The isolated binding member according to claim 38, wherein the first binding domain comprises the amino acid sequence sets SEQ ID NO 17 or a homologue thereof, SEQ ID NO 18 or a homologue thereof, and SEQ ID NO 19 or a homologue thereof, and the amino acid sequence sets SEQ ID NO 21 or a homologue thereof, SEQ ID NO 22 or a homologue thereof, and SEQ ID NO 23 or a homologue thereof.

43. The isolated binding member according to claim 29, wherein the binding member is capable of binding PsaA from two or more different Pneumococcus serotypes.

44. The isolated binding member according to any of claims 38-42, wherein the homologue is at least 60 % homologous to one or more of the sequences, such as at least 65 % homologous such as at least 70 % homologous, such as at least 75 % homologous, such as at least 80 % homologous, such as at least 85 % homologous, such as at least 90 % homologous, such as at least 95 % homologous, such as at least 98 % homologous.

45. The isolated binding member according to claim 29, wherein the first binding domain is capable of specifically binding Streptococcus pneumoniae surface adhesin A (PsaA) protein, said binding domain having a dissociation constant K_d for PsaA which is less than 1×10^{-6} M.

46. The isolated binding member according to any of claims 38-42, wherein the binding domain is located in a V_L domain.

47. The isolated binding member according to any of claims 38-42, wherein the binding domain is located in a V_H domain.

48. The isolated binding member according to any of claims 38-42, wherein the binding domain is arranged as a complementarity-determining region (CDR) in the binding member.

49. The isolated binding member according to claim 29, wherein the fragment of antibodies are selected from Fab, Fab', $F(ab)_2$ and Fv.

50. The isolated binding member according to claim 29, wherein the second binding domain is capable of specifically binding a mammalian protein, such as a human protein, such as a protein selected from CD64 or CD89.

51. The isolated binding member according to claim 29, wherein second binding domain is capable of specifically binding a mammalian cell, such as a human cell, such as a cell selected from a leucocyte, macrophages, lymphocytes, neutrophilic cells, basophilic cells, and eosinophilic cells.

52. The isolated binding member according to claim 29, second binding domain is capable of specifically binding a Pneumococcus protein.

53. The isolated binding member according to claim 29, wherein second binding domain is capable of specifically binding a PsaA epitope different from the first binding domain.

54. The isolated binding member according to claim 29, wherein the binding member comprises two binding domains.

55. The isolated binding member according to claim 54, wherein the two binding members are linked through a spacer region.

56. An isolated nucleic acid molecule encoding at least a part of the binding member as defined in any of claims 1-55.

57. A vector comprising the nucleic acid molecule as defined in claim 56.

58. The vector according to claim 57, comprising a nucleotide sequence which regulates the expression of the antibody encoded by the nucleic acid molecule.

59. A host cell comprising the nucleic acid molecule as defined in claim 56.

60. A cell line engineered to express the binding member as defined in any of claims 1-55.

61. A method of detecting of diagnosing a disease or disorder associated with Pneumococcus in an individual comprising

- providing a biological sample from said individual,
- adding at least one binding member as defined in any of claims 1-55 to said biological sample
- detecting binding members bound to said biological sample, thereby detecting or diagnosing the disease or disorder.

62. A kit comprising at least one binding member as defined in any of claims 1-55, said antibody being labelled.

63. A pharmaceutical composition comprising at least one binding member as defined in any of claims 1-55.

64. The pharmaceutical composition according to claim 63, comprising at least two different binding members.

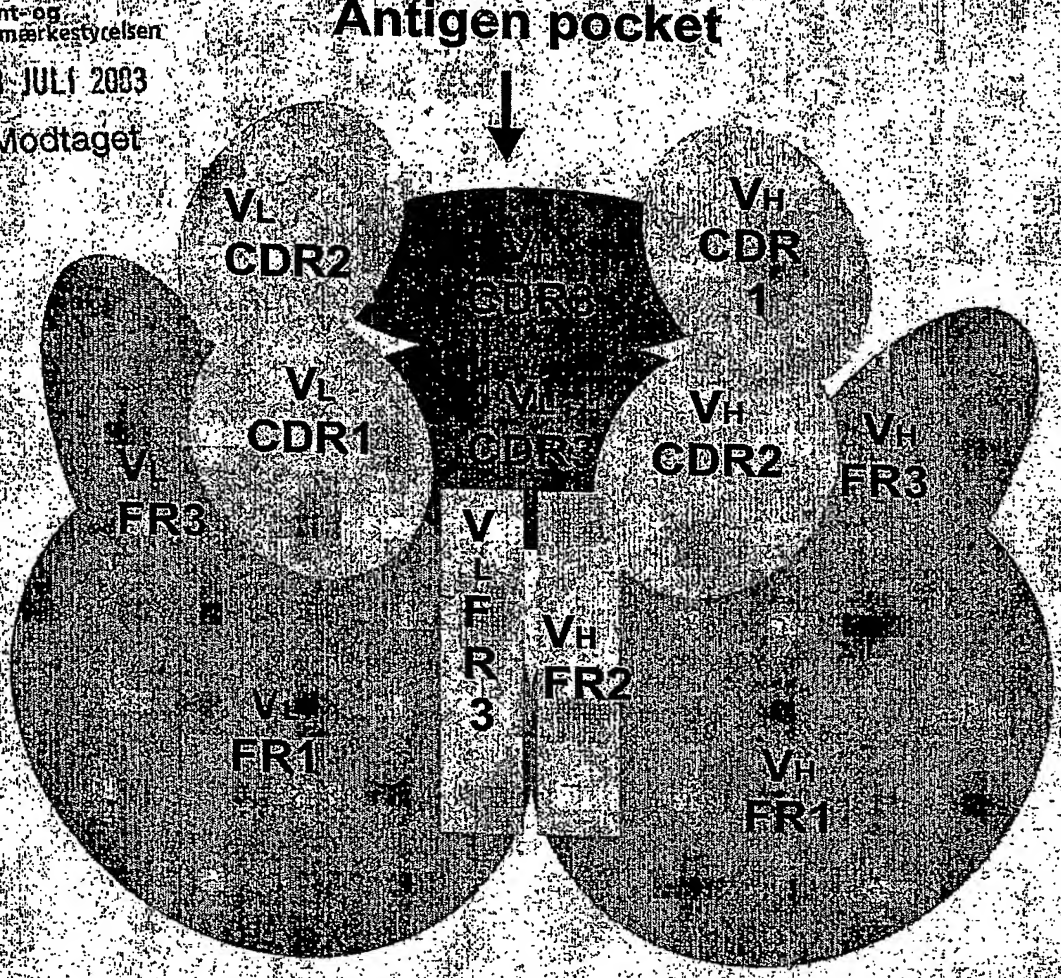
65. Use of a binding member as defined in any of claims 1-55 for the production of a pharmaceutical composition.

66. Use of a binding member as defined in any of claims 1-55 for the production of a pharmaceutical composition for the treatment of pneumonia.

67. A method a treating an individual suffering from pneumonia comprising administering to said indlvidual an effective amount of a binding member as defined in any of claims 1-55.

Fig. 1

Fa



CDR: complementarity determining region
FR: frame region
V_L: variabel light chain
V_H: variabel heavy chain

PA 2003 01044

Fig. 2a

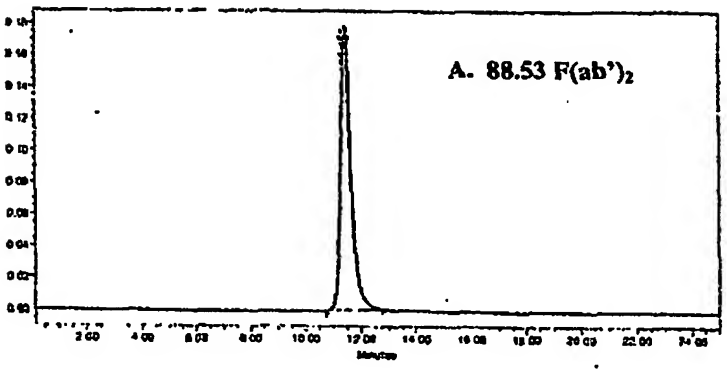


Fig. 2b

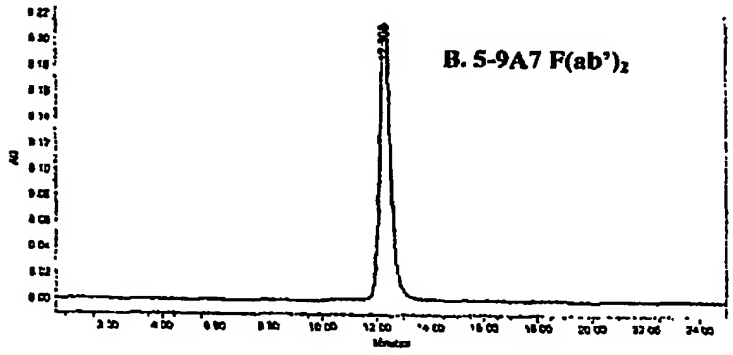


Fig. 3

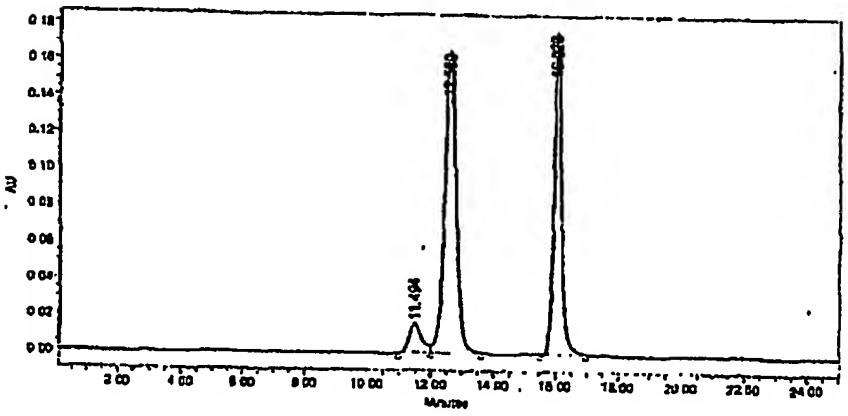


Fig. 4

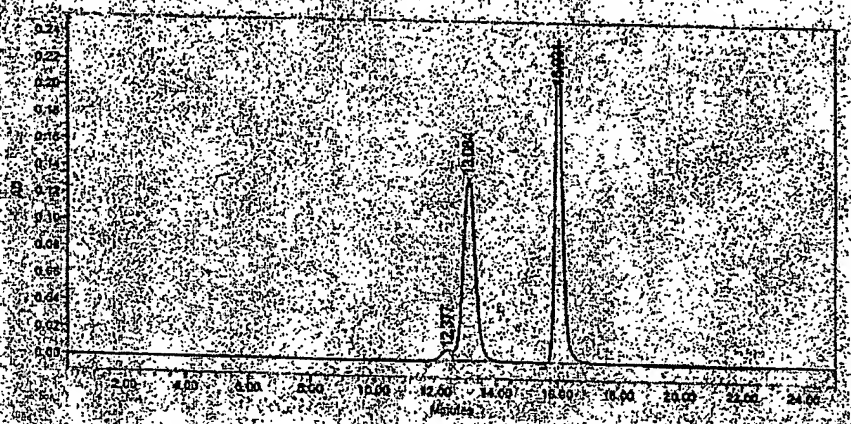


Fig. 5

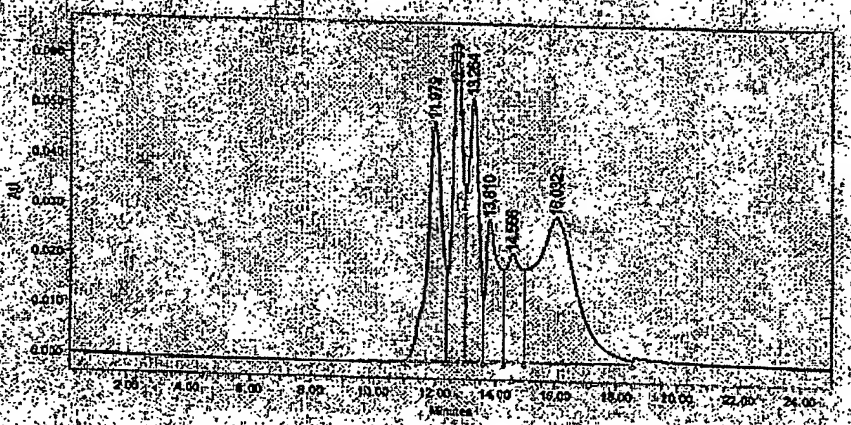


Fig. 6

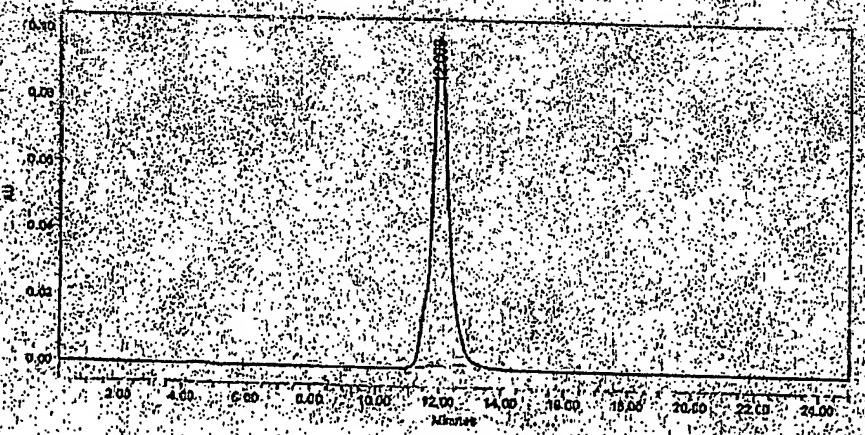


Fig. 7a

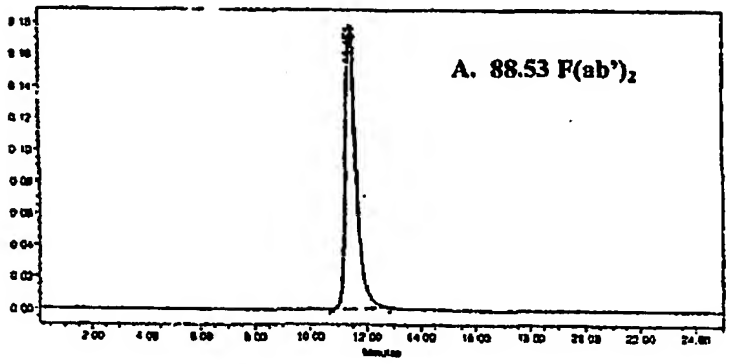


Fig. 7b

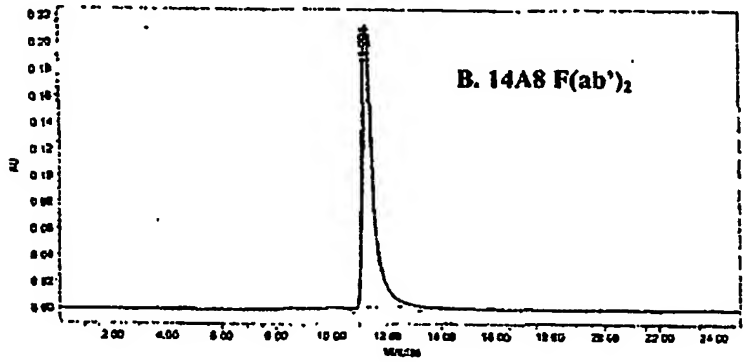


Fig. 8

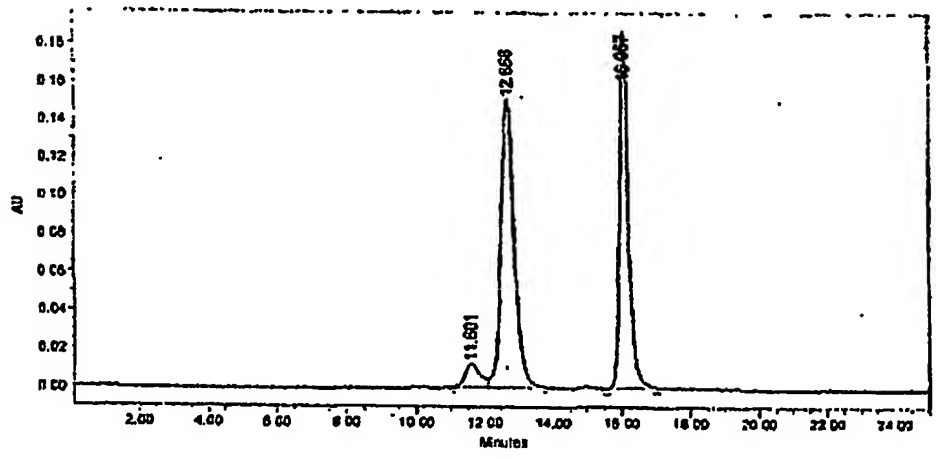


Fig. 9

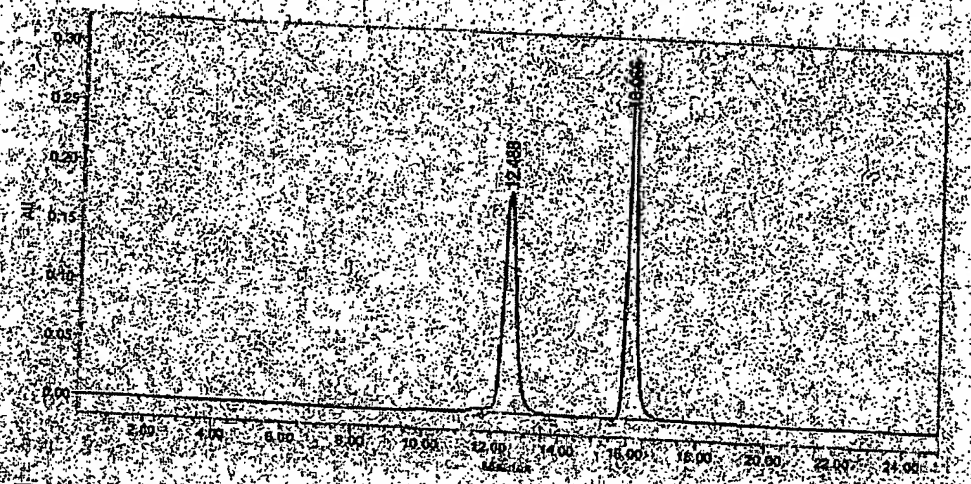


Fig. 10

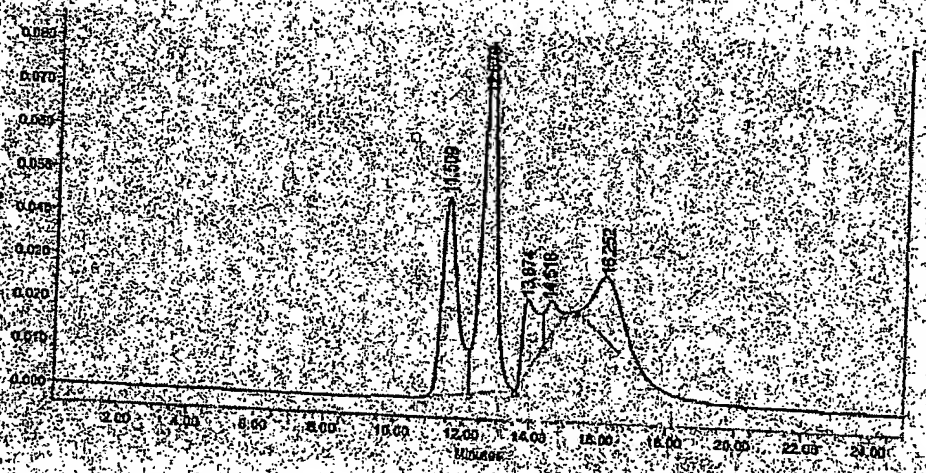


Fig. 11

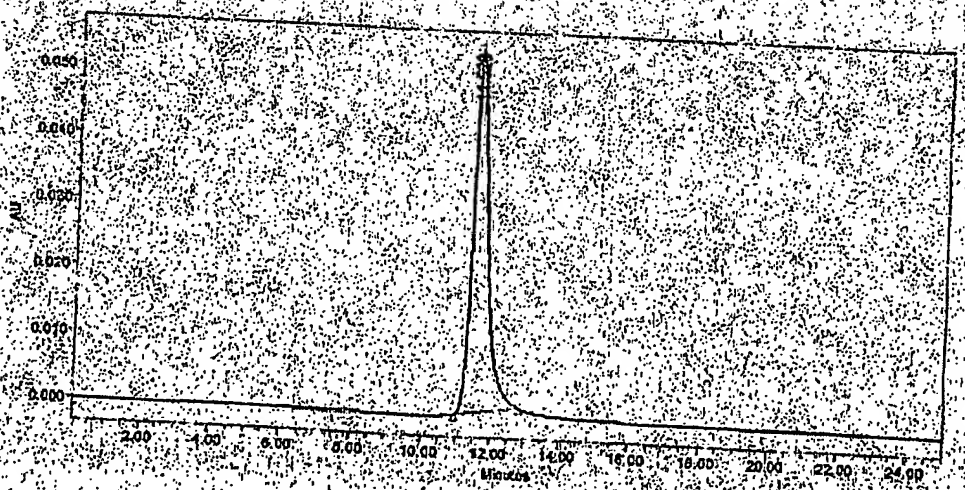


Fig. 12

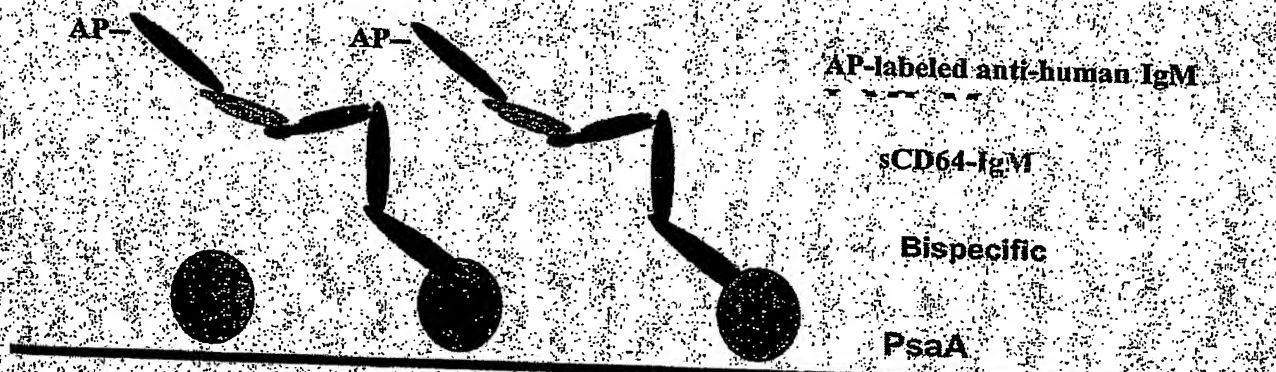


Fig. 13

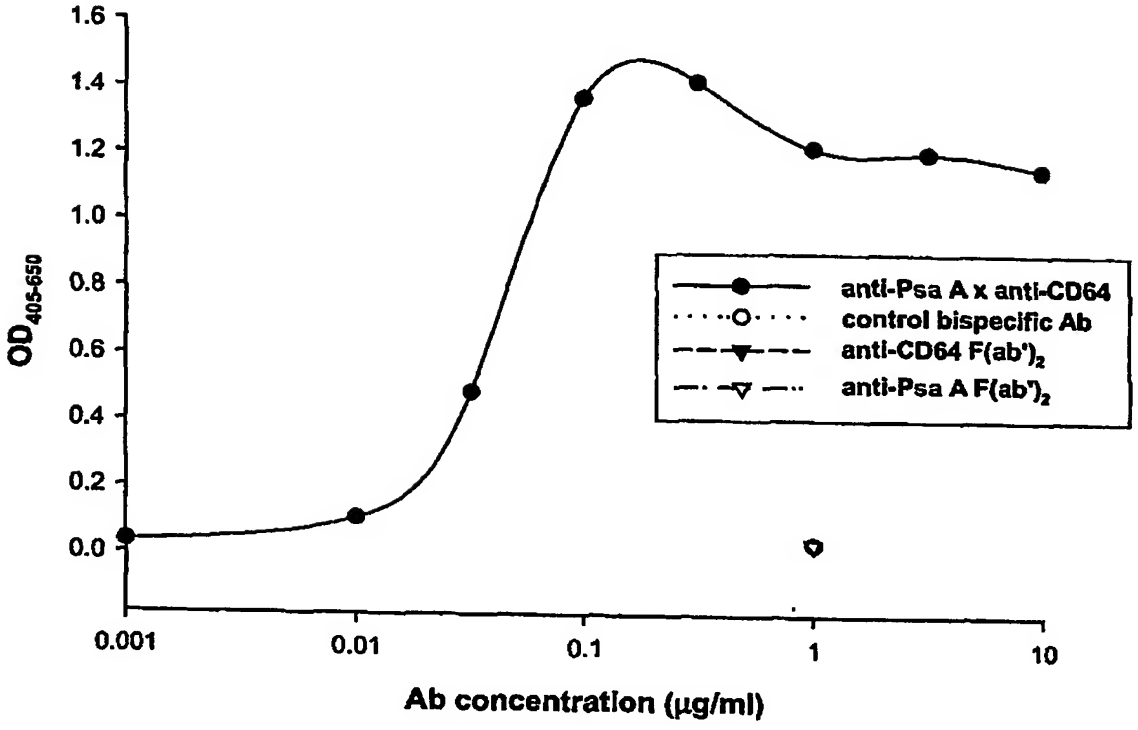


Fig. 14

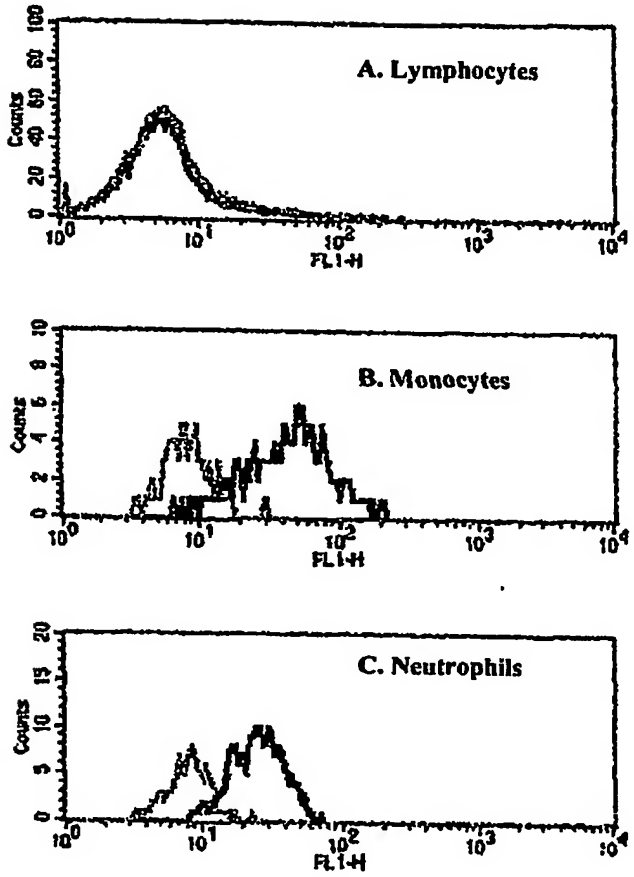


Fig. 15

```
1     ATG AAA AAA TTA GGT ACA TTA CTC GTT CTC TTT CTT TCT GCA ATC    45
1     Met Lys Lys Leu Gly Thr Leu Leu Val Leu Phe Leu Ser Ala Ile    15

46    ATT CTT GTA GCA TGT GCT AGC GGA AAA AAA GAT ACA ACT TCT GGT    90
16    Ile Leu Val Ala Cys Ala Ser Gly Lys Lys Asp Thr Thr Ser Gly    30

91    CAA AAA CTA AAA GTT GTT GCT ACA AAC TCA ATC ATC GCT GAT ATT    135
31    Gln Lys Leu Lys Val Val Ala Thr Asn Ser Ile Ile Ala Asp Ile    45

136   ACT AAA AAT ATT GCT GGT GAC AAA ATT GAC CTT CAT AGT ATC GTT    180
46    Thr Lys Asn Ile Ala Gly Asp Lys Ile Asp Leu His Ser Ile Val    60

181   CCG ATT GGG CAA GAC CCA CAC GAA TAC GAA CCA CTT CCT GAA GAC    225
61    Pro Ile Gly Gln Asp Pro His Glu Tyr Glu Pro Leu Pro Glu Asp    75

226   GTT AAG AAA ACT TCT GAG GCT GAT TTG ATT TTC TAT AAC GGT ATC    270
76    Val Lys Lys Thr Ser Glu Ala Asp Leu Ile Phe Tyr Asn Gly Ile    90

271   AAC CTT GAA ACA GGT GGC AAT GCT TGG TTT ACA AAA TTG GTA GAA    315
91    Asn Leu Glu Thr Gly Gly Asn Ala Trp Phe Thr Lys Leu Val Glu    105

316   AAT GCC AAG AAA ACT GAA AAC AAA GAC TAC TTC GCA GTC AGC GAC    360
106   Asn Ala Lys Lys Thr Glu Asn Lys Asp Tyr Phe Ala Val Ser Asp    120

361   GGC GTT GAT GTT ATC TAC CTT GAA GGT CAA AAT GAA AAA GGA AAA    405
121   Gly Val Asp Val Ile Tyr Leu Glu Gly Gln Asn Glu Lys Gly Lys    135

406   GAA GAC CCA CAC GCT TGG CTT AAC CTT GAA AAC GGT ATT ATT TTT    450
136   Glu Asp Pro His Ala Trp Leu Asn Leu Glu Asn Gly Ile Ile Phe    150

451   GCT AAA AAT ATC GCC AAA CAA TTG AGC GCC AAA GAC CCT AAC AAT    495
151   Ala Lys Asn Ile Ala Lys Gln Leu Ser Ala Lys Asp Pro Asn Asn    165

496   AAA GAA TTC TAT GAA AAA AAT CTC AAA GAA TAT ACT GAT AAG TTA    540
166   Lys Glu Phe Tyr Glu Lys Asn Leu Lys Glu Tyr Thr Asp Lys Leu    180

541   GAC AAA CTT GAT AAA GAA AGT AAG GAT AAA TTT AAT AAG ATC CCT    585
181   Asp Lys Leu Asp Lys Glu Ser Lys Asp Lys Phe Asn Lys Ile Pro    195

586   GCT GAA AAG AAA CTC ATT GTA ACC AGC GAA GGA GCA TTC AAA TAC    630
196   Ala Glu Lys Lys Leu Ile Val Thr Ser Glu Gly Ala Phe Lys Tyr    210

631   TTC TCT AAA GCC TAT GGT GTT CCA AGT GCC TAC ATC TGG GAA ATC    675
211   Phe Ser Lys Ala Tyr Gly Val Pro Ser Ala Tyr Ile Trp Glu Ile    225

676   AAT ACT GAA GAA GAA GGA ACT CCT GAA CAA ATC AAG ACC TTG GTT    720
226   Asn Thr Glu Glu Glu Gly Thr Pro Glu Gln Ile Lys Thr Leu Val    240

721   GAA AAA CTT CGC CAA ACA AAA GTT CCA TCA CTC TTT GTA GAA TCA    765
241   Glu Lys Leu Arg Gln Thr Lys Val Pro Ser Leu Phe Val Glu Ser    255

766   AGT GTG GAT GAC CGT CCA ATG AAA ACT GTT TCT CAA GAC ACA AAC    810
256   Ser Val Asp Asp Arg Pro Met Lys Thr Val Ser Gln Asp Thr Asn    270

811   ATC CCA ATC TAC GCA CAA ATC TTT ACT GAC TCT ATC GCA GAA CAA    855
271   Ile Pro Ile Tyr Ala Gln Ile Phe Thr Asp Ser Ile Ala Glu Gln    285

856   GGT AAA GAA GGC GAC AGC TAC TAC AGC ATG ATG AAA TAC AAC CTT    900
286   Gly Lys Glu Gly Asp Ser Tyr Tyr Ser Met Met Lys Tyr Asn Leu    300

901   GAC AAG ATT GCT GAA GGA TTG GCA AAA TAA    930
301   Asp Lys Ile Ala Glu Gly Leu Ala Lys End
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Fig. 16a

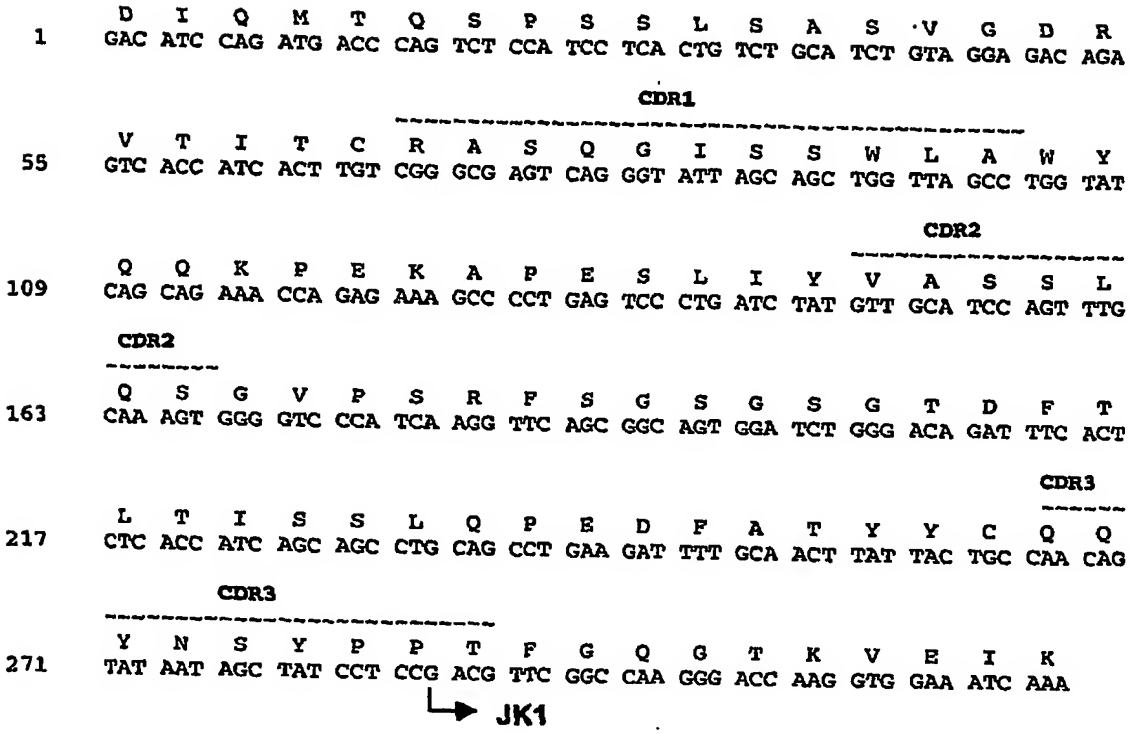
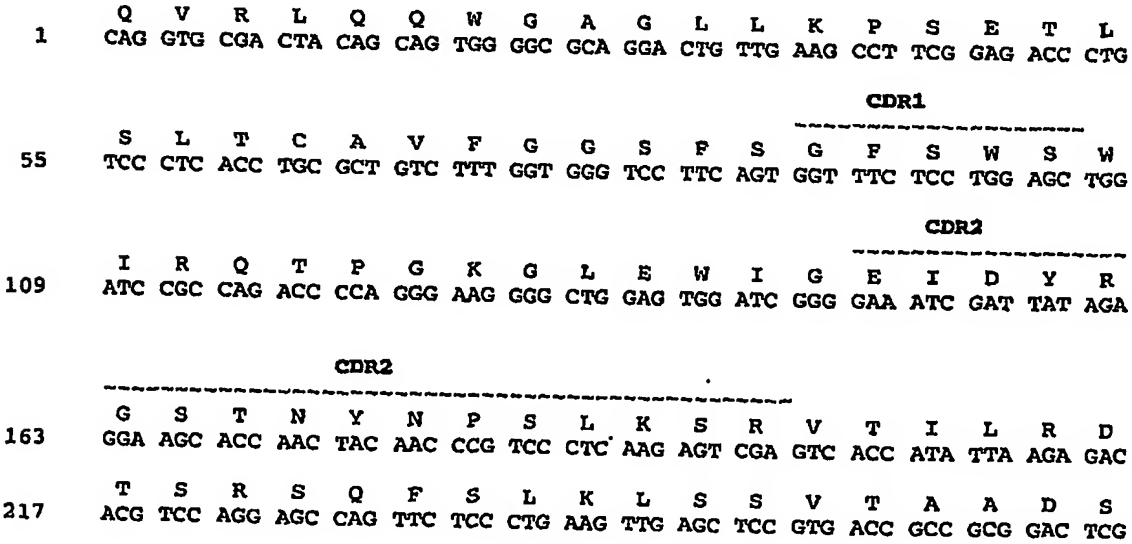


Fig. 16b



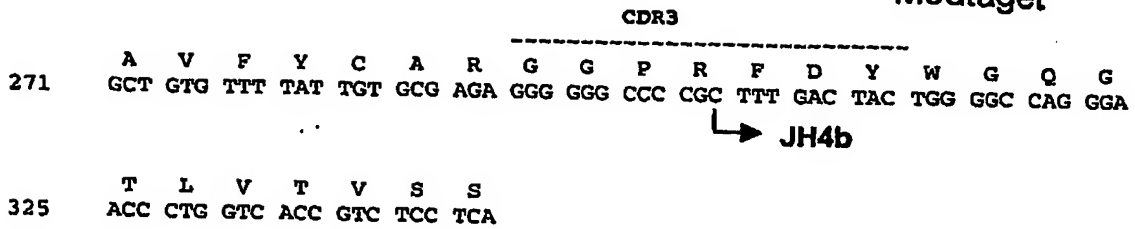


Fig. 17a

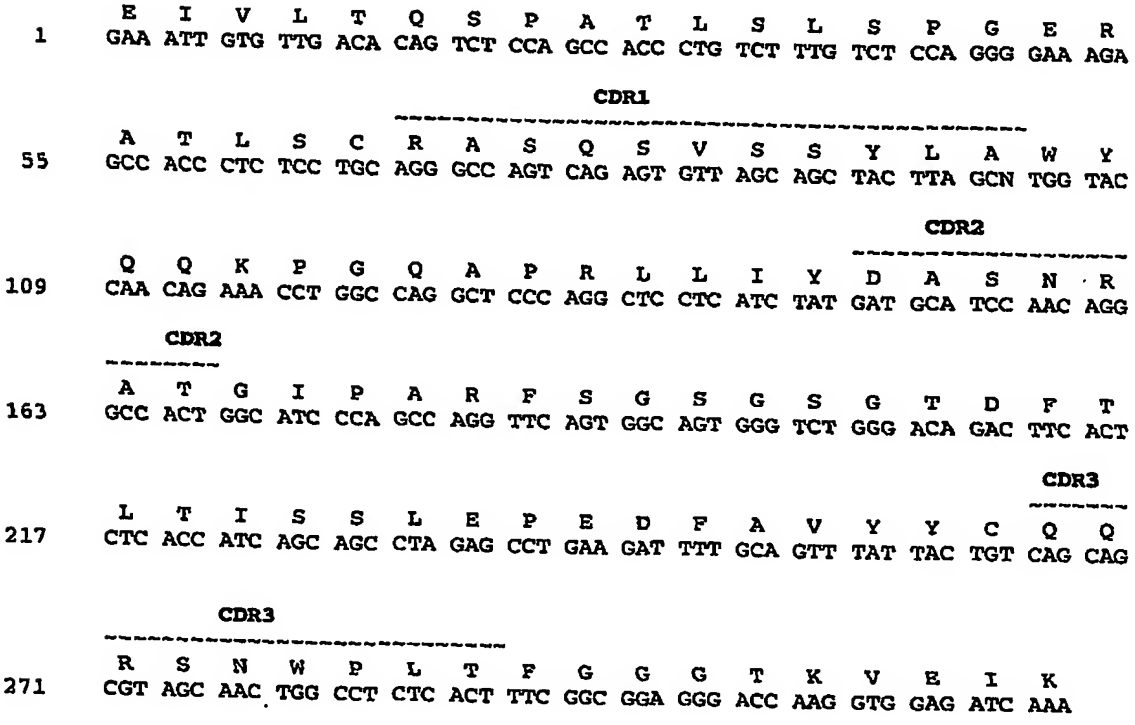
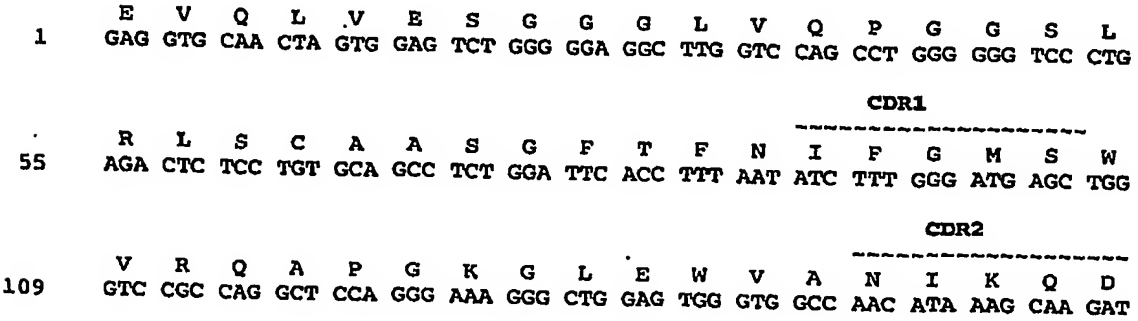


Fig. 17b



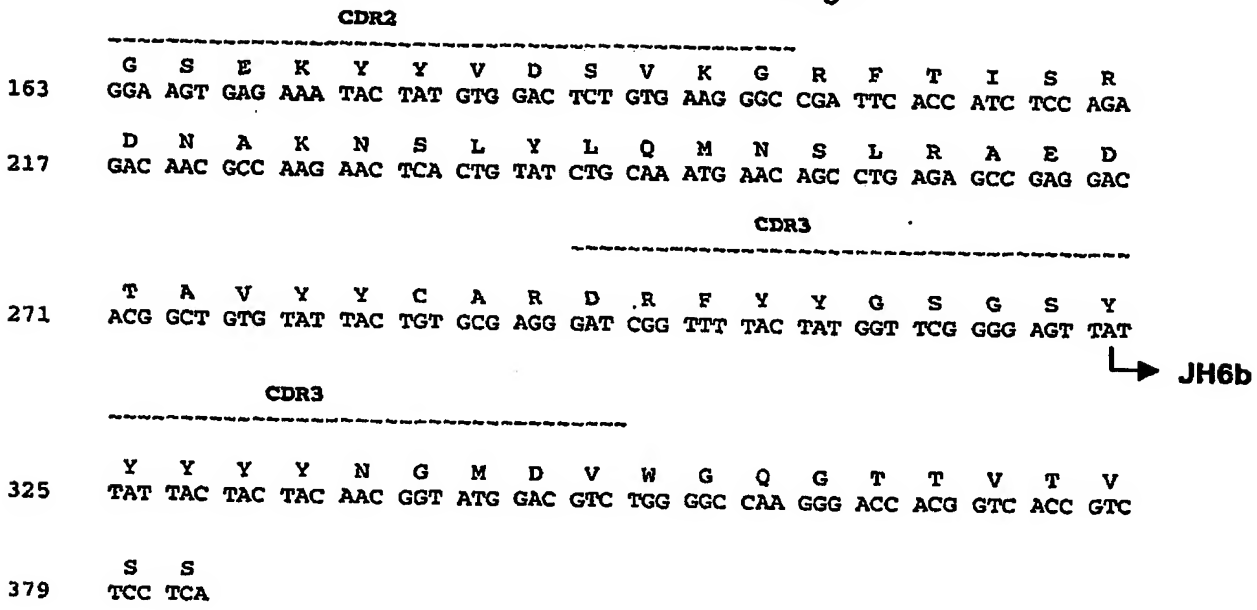


Fig. 18a

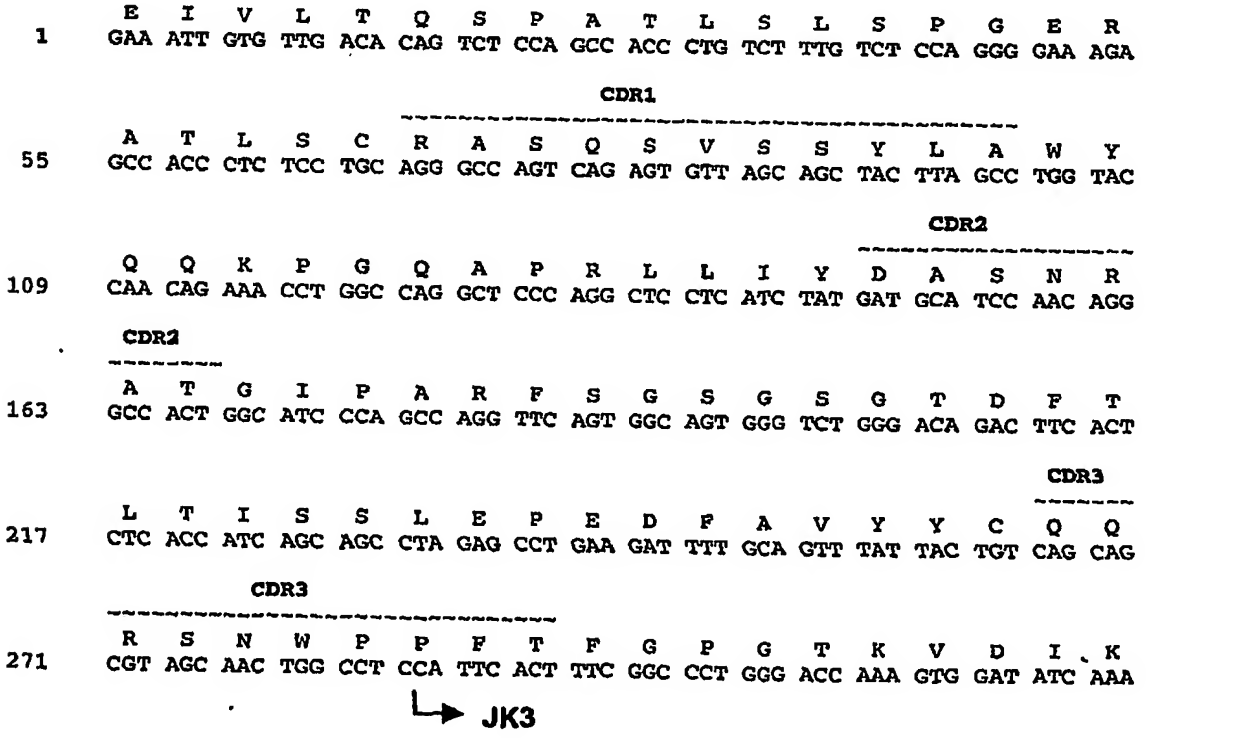


Fig. 18b

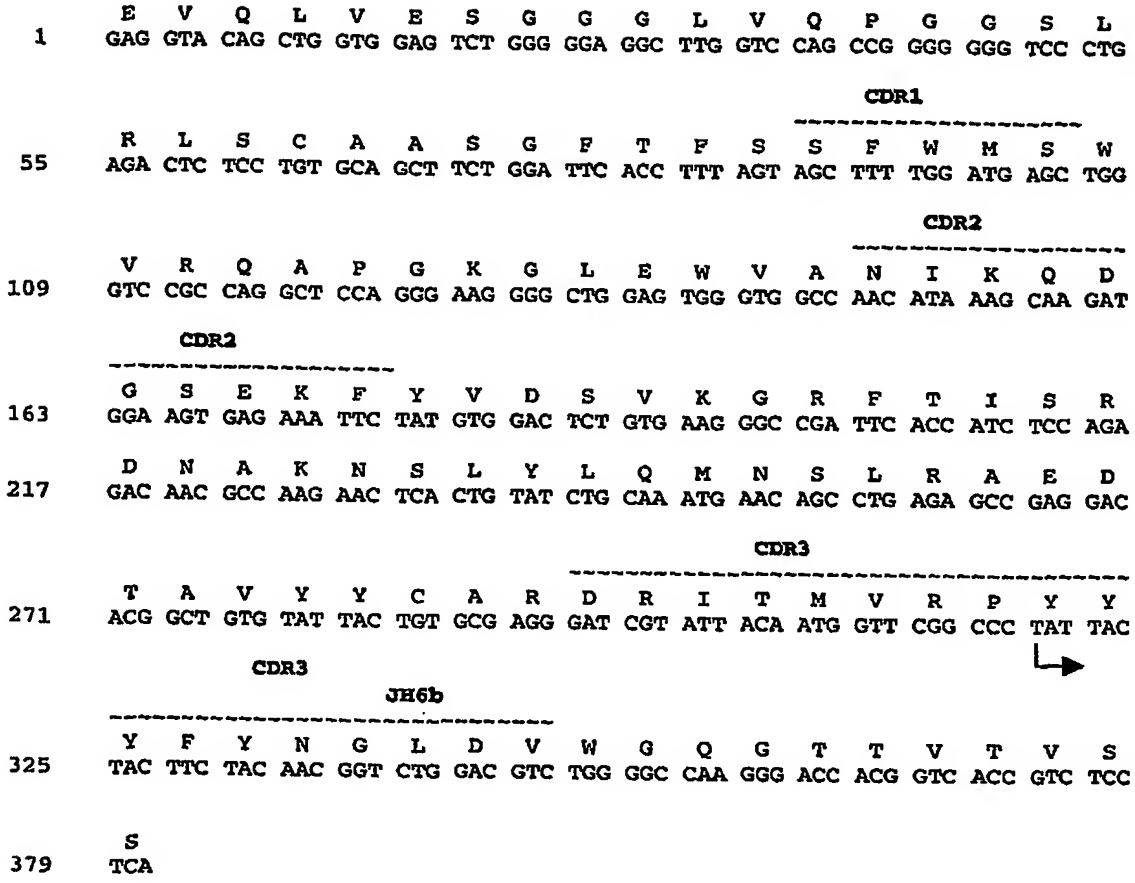
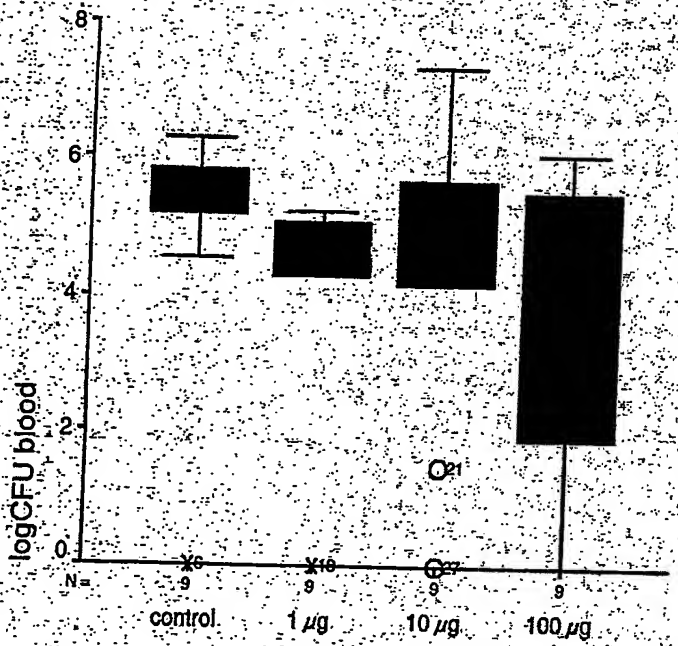


Figure 19A

9A7

Blood (logCFU)



AGRP

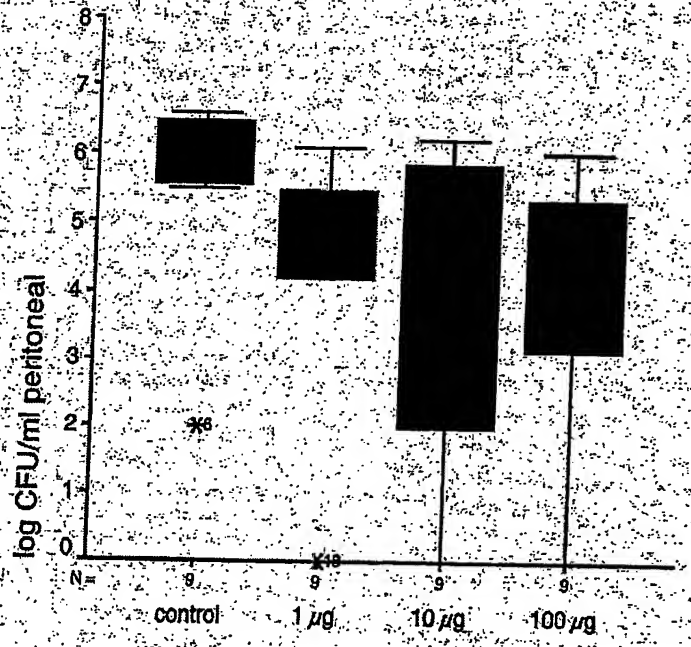
Test Statistics[a,b]

	CFU/ml blood
Chi-Square	4,711
df	3
Asymp. Sig.	,194

a. Kruskal Wallis Test

b. Grouping Variable: AGRP

Peritoneal (logCFU)



AGRP

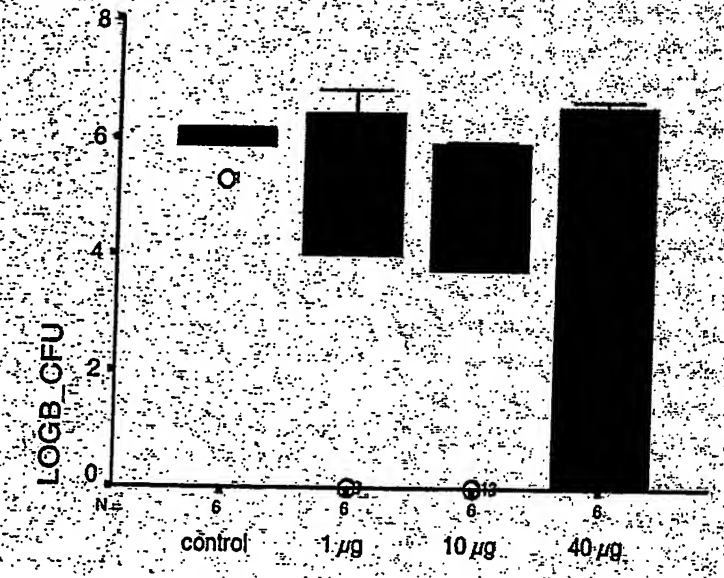
Test Statistics[a,b]

	CFU/ml peritoneal
Chi-Square	7,926
df	3
Asymp. Sig.	,048

a. Kruskal Wallis Test

b. Grouping Variable: AGRP

Figure 19B
15E5

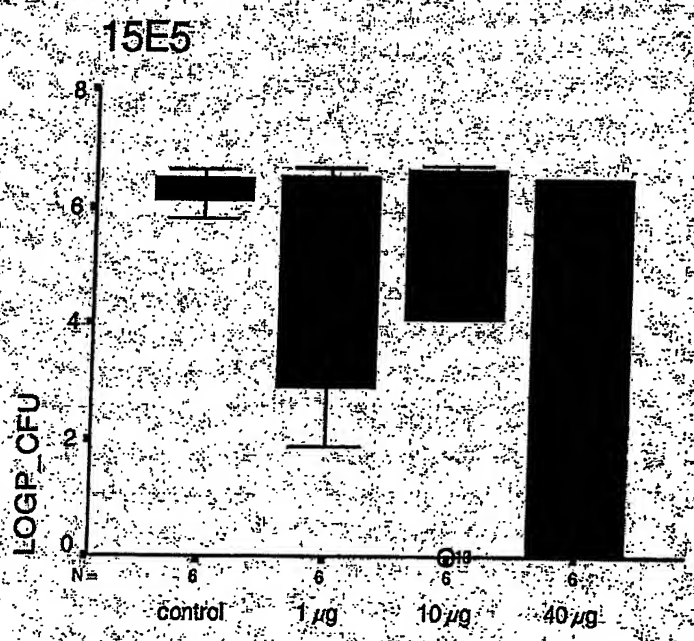


Test Statistics[a,b]

	B_CFU
Chi-Square	2,493
df	3
Asymp. Sig.	,477

a. Kruskal Wallis Test

b. Grouping Variable: AGRP



Test Statistics[a,b]

	P_CFU
Chi-Square	,532
df	3
Asymp. Sig.	,912

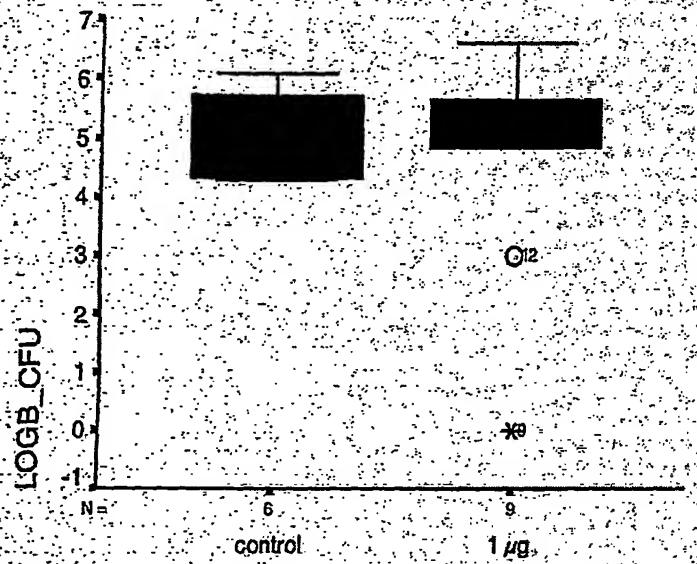
a. Kruskal Wallis Test

b. Grouping Variable: AGRP

Figure 19C
1G9

Blood (log CFU)

1G9.1



Ab

Test Statistics[b]

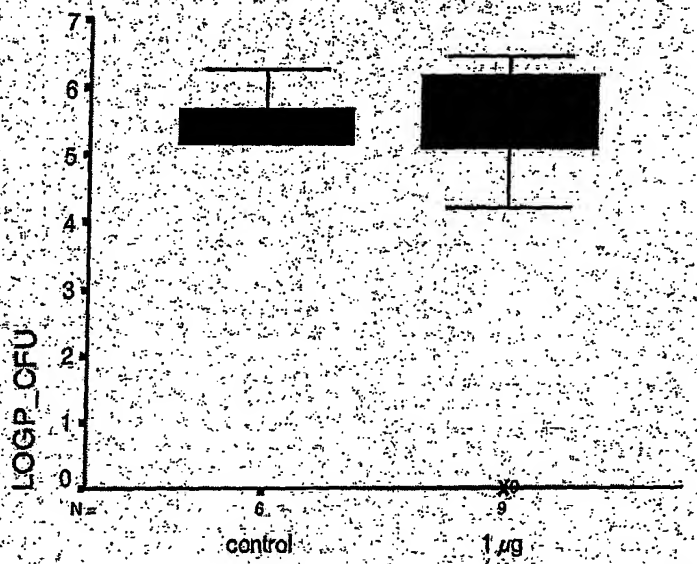
	B_CFU
Mann-Whitney U	23,000
Wilcoxon W	44,000
Z	-,471
Asymp. Sig. (2-tailed)	,637
Exact Sig. [2*(1-tailed Sig.)]	,689[a]

a. Not corrected for ties.

b. Grouping Variable: AGRP

Peritoneal (log CFU)

1G9.1



Ab

Test Statistics[b]

	P_CFU
Mann-Whitney U	22,500
Wilcoxon W	43,500
Z	-,531
Asymp. Sig. (2-tailed)	,596
Exact Sig. [2*(1-tailed Sig.)]	,607[a]

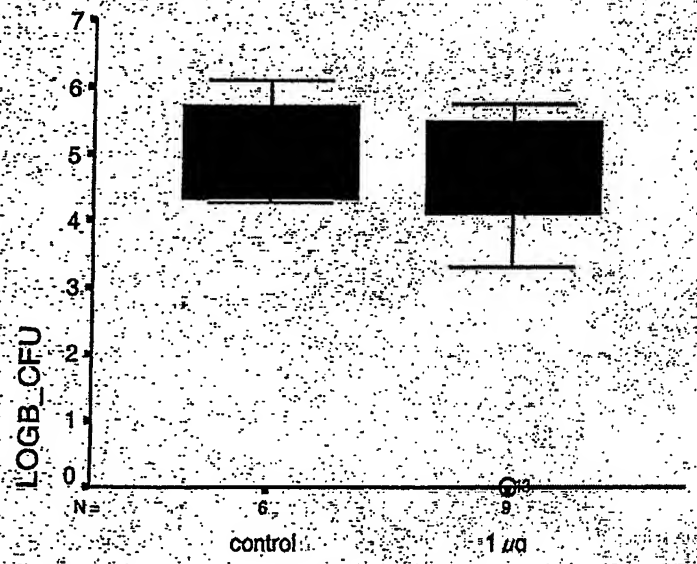
a. Not corrected for ties.

b. Grouping Variable: AGRP

Figure 19D
3D10

Blood (log CFU)

3D10



Ab

Test Statistics[b]

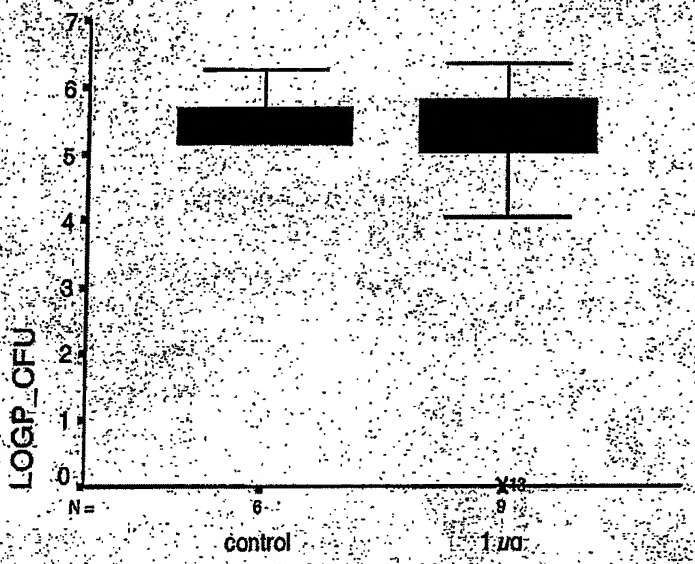
	B_CFU
Mann-Whitney U	23,000
Wilcoxon W	68,000
Z	-,471
Asymp. Sig. (2-tailed)	,637
Exact Sig. [2*(1-tailed Sig.)]	,689[a]

a. Not corrected for ties.

b. Grouping Variable: AGRP

Peritoneal (log CFU)

3D10



Ab

Test Statistics[b]

	P_CFU
Mann-Whitney U	27,000
Wilcoxon W	72,000
Z	,000
Asymp. Sig. (2-tailed)	1,000
Exact Sig. [2*(1-tailed Sig.)]	1,000[a]

a. Not corrected for ties.

b. Grouping Variable: AGRP

Figure 19E

% reduction in mean CFU/ml from control (9A7)

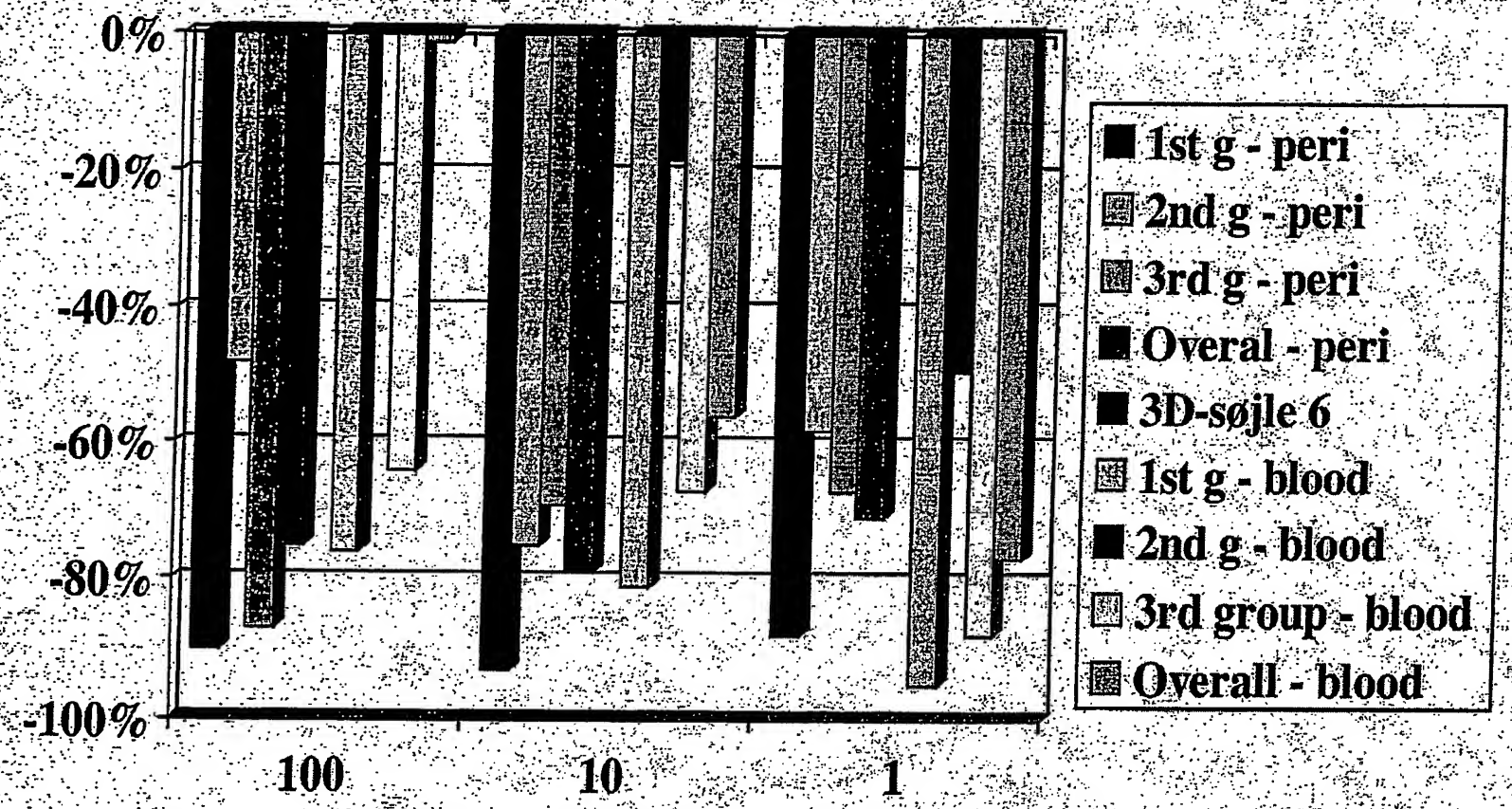


Fig. 20

Clone	Sub type	c1q	R6 ELISA (corrected)					R6 WB	ELISA (0.5 µg/ml) (corrected)												Western Blot (2 µg/ml)								
			5 µg/ml	0.5 µg/ml	50 ng/ml	5 ng/ml	0.5 ng/ml	2 µg/ml	R6	1	3	4	6B	7F	8	9V	12F.	14	23F	D39	R6	1	3	4	6B	7F	8	9V	12F
1-7D12	G3		1,58	0,35	0,23	0,20	0,20	0	0,42	0,45	0,38	0,40	0,60	0,49	0,55	0,44	0,51	0,38	0,40	1,23	0	0	0	0	0	0	0	0	0
1-7H7	G3		0,00	0,50	0,22	0,19	0,20	0	0,38	0,52	0,40	0,49	0,64	0,53	0,62	0,50	0,60	0,39	0,44	0,69	0	0	0	0	0	0	0	0	0
1-11H10	G3		0,00	0,86	0,52	0,26	0,22	1	0,90	0,30	0,79	0,26	0,39	0,81	1,08	2,56	1,10	0,77	0,24	0,06	1	1	1	1	1	1	1	1	1
1-12E10	G3		2,01	0,42	0,24	0,21	0,21	0	0,56	0,47	0,29	0,31	0,48	0,51	0,46	0,55	0,50	0,38	0,37	0,83	0	0	0	0	0	0	0	0	0
1-15E5	G1		1,74	1,71	0,79	0,30	0,25	1	0,99	0,31	0,97	0,30	0,37	0,96	1,19	2,49	1,09	1,09	0,33	0,68	1	1	1	1	1	1	1	1	1
1-15E5.2.1	G1		1,33	1,69	1,53	0,55	0,32	1	1,20	0,36	1,06	0,35	0,35	1,06	1,33	>3	1,23	1,14	0,34	0,65	1	1	1	1	1	1	1	1	1
3-4F10	G1		0,98	0,38	0,24	0,21	0,17	1	0,45	0,53	0,37	0,60	0,59	0,46	0,42	0,75	0,38	0,66	0,41	0,72	1	1	1	1	(0)	1	1	1	1
4-3D10	G3		1,23	1,19	0,79	0,33	0,25	1	0,90	0,33	0,92	0,33	0,44	0,74	0,95	2,31	1,06	0,92	0,37	0,91	1	1	1	1	(0)	1	1	1	1
4-4B11	G3		1,23	0,90	0,49	0,29	0,26	1	0,83	0,36	0,79	0,34	0,37	0,66	0,87	1,93	0,86	0,67	0,29	0,81	1	1	1	1	(0)	1	1	1	1
4-7E8	G3		0,00	-0,45	0,29	0,27	0,28	1	-1,46	-2,00	-1,46	-1,96	-1,94	-1,55	-1,28	-0,23	-1,23	-1,40	-1,97	-1,23	1	1	1	1	(0)	1	1	1	1
4-7H8	G3		1,45	1,29	0,54	0,31	0,27	1	0,74	0,26	0,66	0,20	0,31	0,63	0,84	2,04	0,87	0,66	0,28	0,58	1	1	1	1	(0)	1	1	1	1
4-10G6	G1		0,97	0,35	0,25	0,24	0,25	0	0,32	0,07	0,24	0,07	0,19	0,29	0,35	0,36	0,36	0,25	0,10	0,41	1	1	1	1	(0)	1	1	1	1
5-2G6	G1		0,58	0,42	0,31	0,25	0,25	1	0,47	0,07	0,27	0,06	0,18	0,29	0,40	1,05	0,38	0,20	0,09	0,38	1	1	1	1	(0)	1	1	1	1
5-2G8	G1		1,57	1,61	0,80	0,33	0,27	1	1,38	0,35	1,18	0,28	0,34	0,93	1,24	2,83	1,27	1,05	0,30	0,62	1	1	1	1	1	1	1	1	1
5-5E10	G1		1,74	1,59	0,57	0,29	0,26	1	1,33	0,31	1,28	0,30	0,34	0,93	1,33	2,74	1,24	1,03	0,31	0,59	1	1	1	1	1	1	1	1	1
5-9A7	G1		1,39	1,65	1,32	0,41	0,26	1	1,39	0,40	1,31	0,33	0,39	1,17	1,52	>3	1,51	1,42	0,37	0,66	1	1	1	1	1	1	1	1	1
5-9C7	G1		1,65	0,88	0,35	0,26	0,24	1	1,14	0,26	0,86	0,20	0,33	0,82	1,12	2,29	1,10	0,95	0,27	0,65	1	1	1	1	1	1	1	1	1
5-10G9	G1		0,97	0,77	0,36	0,26	0,26	1	0,25	0,03	0,02	0,03	0,16	0,18	0,19	0,13	0,17	0,05	0,06	0,40	1	1	1	1	1	1	1	1	1
6-7A4	G1		1,38	1,69	1,08	0,43	0,28	1	1,20	0,24	0,96	0,19	0,29	0,71	1,11	2,66	1,04	0,85	0,25	0,63	1	1	1	1	1	1	1	1	1
6-10E5	G1		-0,11	1,12	0,86	0,33	0,22	1	1,32	0,35	1,14	0,32	0,39	1,05	1,44	>3	1,44	1,41	0,38	0,52	1	1	1	1	1	1	1	1	1
7-1G9.1	G1		1,36	1,68	1,78	0,54	0,31	1	1,38	0,35	1,24	0,33	0,40	1,06	1,50	>3	1,46	1,32	0,36	0,68	1	1	1	1	1	1	1	1	1
7-7F12.1	G1		0,00	1,01	1,08	0,39	0,27	1	1,06	0,25	0,99	0,27	0,37	0,94	1,15	>3	1,27	1,06	0,31	0,39	1	1	1	1	1	1	1	1	1
7-9E2.1	G1		0,00	1,10	1,18	0,47	0,29	1	1,01	0,28	0,91	0,24	0,29	0,93	1,13	2,46	1,30	1,05	0,29	0,45	1	1	1	1	1	1	1	1	1